CA^{2+}-INDUCED SPORULATION IN THE FUNGUS ACHLYA: THE BIOLOGICAL ROLES OF β-(1->3)-GLUCANS AND KEY ENZYMES

By

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In Memory of My Mother

and

To My Baby and My Wife

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This research utilized the fungus Achlya under growth conditions which caused the development of asexual reproductive structures termed sporangia. Achlya accumulates, under optimal growth conditions, reserve β-(1->3)-glucans which contain phosphate and glucose. These reserves represent 14 % of the dry weight of the fungal mycelium, and the phosphate is linked to the glucose by both mono- and diphosphoester bonds.

The first series of experiments defined cultural conditions which produced the highest activities of the several enzymes which are required to hydrolyze the reserve β-(1->3)-glucans releasing both glucose and phosphate. It was found that the concentrations of phosphate and carbon supplied in the cultural medium greatly influenced the activities of these enzymes. The highest activities of phosphodiesterase and phosphomonoesterase were found with the lowest concentration of phosphate (0.015 mM). When the glucose supply was limited, the highest

activity of β-(1->3)-glucanase was found. A β-(1->3)-glucanase, apparently regulated by the concentration of phosphate in the presence of glucose rather than by glucose alone, was found.

One of the β-(1->3)-glucanase enzymes was purified and characterized. This is an exo-type enzyme producing glucose when the reserve β-(1->3)-glucans were used as substrates. Low activity was obtained when the phosphoglucan was used as substrate.

The final series of experiments involved Ca^{2+}- induced sporulation which brought together the enzyme experiments and the utilization of the reserve β-(1->3)-glucans. The process of sporulation can be observed under the microscope and consists of distinctive morphological events over about ten hours. It converts about 12 % of the mycelial dry weight into spores and reduces the reserve β-(1->3)-glucans by 49 %. Phosphate is released from the reserve β-(1->3)-glucans by the action of phosphodiesterase and phosphomonoesterase as required by the two types of ester linkages. This makes available the neutral glucan which was found to be the best substrate for the β-(1->3)-glucanase. The ratio of the mono- and diester linkages, and the molecular size of the phosphoglucan did not change based on results from both gel filtration and ^{31}P-NMR, suggesting that there is no preferential cleavage of the diester bonds. A model for this cooperation enzyme action is presented.

Chapter 1
INTRODUCTION

Achlya bisexualis is an aquatic species of the class Oomycetes, whose members commonly live in soil or fresh water. A major point of interest is the complex sexual cycle which is initiated and sequentially controlled by a series of steroid hormones, which are secreted by compatible mating types (Mullins, 1994).

When mycelium of Achlya bisexualis is cultured in a complete medium, it will grow vigorously and accumulate cytoplasmic polysaccharides as reserve materials (Faro, 1972; Cottingham and Mullins, 1985; Lee and Mullins, 1994). These polysaccharides were recently shown to be β-(1->3) linked polymers of two types: a small neutral form (about 20 %) and a large phosphorylated form (about 80 %), containing both mono- and diphosphoester linkages (Lee et al., 1996). ^{13}C NMR spectra of the neutral and phosphorylated glucans are qualitatively similar. Aqueous hydrofluoric acid cleaves the phosphate from the polymer and produces molecules of the size and structure of the neutral form. Thus, it was proposed that phosphodiester covalent bonds aggregate the neutral form into the very large phosphoglucan. Similar β-(1->3)-glucans have been isolated and characterized from other species of Oomycetes (Zenvenhuizen and Bartnicki-Garcia, 1970; Coulter and Aronson, 1977; Wang and Bartnicki-Garcia, 1980), and they appear to be the major storage polysaccharide in this class.

Nutrient limitation is a form of stress organisms frequently experience, and a common response is the utilization of reserve materials. This utilization is made possible by the activation of appropriate hydrolytic enzymes. Phosphorus and carbon are two essential nutrients, and the relationship between hydrolytic enzyme activities and limitation of these two elements has been widely studied (Lilley and Ball, 1974; Friebe and Halldorf, 1975; Stantos et al., 1977; 1978; Hill and Mullins, 1979; Rosa et al., 1984; Kitamoto et al., 1987; Vinter et al., 1987; Salt and Gander, 1988; Rapp, 1989; Rasmussen et al., 1992; Seeger and Jerez, 1993; Jensen et al., 1993; Scholten et al., 1995). Far less known is the interaction between these two elements, but this was possible in this study because of the structure of the cytoplasmic β-(1->3)-glucans of Achlya.

Sporulation is a major morphological event, not only for Achlya but also for most fungi. It is frequently initiated by nutrient limitation and involves specific genes and their products.

The primary goal of the present study is to utilize the fungus Achlya bisexualis and determine the biological role(s) of the cytoplasmic β-(1->3)-glucans and associated hydrolytic enzymes. Sporulation was chosen because it is a clearly defined morphological event, involving rapid biochemical changes. In addition, information from the enzymatic hydrolysis of the β-(1->3)-glucans may provide the groundwork for elucidating a more detailed structure of these molecules.

Chapter 2
REVIEW OF LITERATURE

The Organism, Achlya bisexualis

Achlya bisexualis, ATCC accession number 14524, belongs to the order Saprolegniales, class Oomycetes, subdivision Mastigomycotina, Division Eumycota of the Kingdom Fungi (Carlile and Watkinson, 1994). The subdivision Mastigomycotina brings together organisms having motile spores (zoospores) or gametes, or those which are closely related on other characters. This subdivision contains three classes: Chytridiomycetes, Hypochytriomycetes and Oomycetes. The first is related to other Eumycota, while the last two are more closely related to certain protistan groups rather than to other fungi.

The Oomycetes, of which about 600 species are known, consist of 4 orders: Saprolegniales, Lagenidiales, Peronosporales and Leptomitales (Hawksworth et al., 1994).

The sexual phase of the Oomycetes has a clear differentiation into large female and small male structures, termed oogonium and antheridium, and these are the sites of meiosis and gametogenesis. Each oospore, produced by fertilization, has a single diploid nucleus. When the oospore germinates, it gives rise to a vegetative mycelium that is diploid, in contrast to the haploid mycelium of most fungi. Another characteristic of the Oomycetes that distinguishes them from the Eumycota is the biflagellate zoospore (Carlile and Watkinson, 1994).

distinguishes them from the Eumycota is the biflagellate zoospore (Carlile and Watkinson, 1994).

Members of the order Saprolegniales, often referred to as the water molds, are common in fresh water and damp soil, most living as saprotrophes, and a few are parasitic. Achlya, as a member of this order, exhibits an absorptive, heterotrophic mode of nutrition plus a conspicuous cell wall of cellulose and a mycelial vegetative state.

Achlya bisexualis is filamentous, the thallus consists of branched coenocytic filaments called hyphae, or collectively the mycelium. Cross walls are produced to delimit reproductive structures, and vegetative growth occurs at the apex.

Achlya is capable of both sexual and asexual reproduction. Asexually, individuals reproduce by fragmentation, by differentiation of hyphae into resistant gemmae, or by differentiation of vegetative apices into clavate zoosporangia separated from the rest of hyphae by cross walls (Johnson, 1956). During asexual sporulation, sporangium production can be initiated by starvation conditions (Carlile and Watkinson, 1994). Zoospores form inside the sporangium, and have an anterior tinsel type and a posterior whiplash flagellum.

Achlya differs from related genera in that their primary zoospores encyst immediately upon discharge, forming at the exit pore of the sporangium a cluster of cysts from which secondary zoospores emerge (Johnson, 1956). A period of swimming occurs followed by another encystment. Given a suitable substrate, the encysted spore will germinate via a germ tube and produce a hyphal system.

Most water molds are monoecious, bearing both male and female reproductive structures on a single diploid mycelium. Achlya bisexualis is one of a few dioecious species that consist of compatible mating types. The sexual cycle utilizes meiosis and gametogenesis

to produce haploid gametes, which rapidly fuse to produce the diploid state. Sexual reproduction is initiated and sequentially controlled by a series of diffusible hormones. Raper (1940) first described this hormonal mechanism in Achlya, where vegetative female hyphae secrete a steroid hormone, now named antheridiol, into the growth medium, and hyphae of male strains take up and respond to this hormone by forming numerous antheridial branches. These sexually induced male strains then secrete another hormone, now named oogoniol, which induces the female to develop oogonial initials. Antheridial hyphae grow, probably following a hormone gradient, toward the oogonial initials. Upon making contact, sexual development is completed by the formation of cross walls which delimit the antheridium and the oogonium. Meiosis and gametogenesis then occur and fertilization follows via a fertilization tube from the antheridium into the oogonium. Oospores or zygotes are thus formed inside the oogonium.

Hydrolytic Enzymes and Nutrient Limitation

Organisms, especially microorganisms, frequently confront a limitation of nutrients. This is a form of stress which normally results in the cessation of exponential growth, or in the initiation of reproductive cycles. Hydrolytic enzymes are activated, often at the gene level, in order to provide necessary products for survival.

Phosphorus is an essential element in all cells, playing a vital functional role in energy metabolism and as a structural constituent of many biomolecules (Duff et al., 1994). Inorganic phosphate is transported into cells where it enters the organophosphate pool. In

some bacteria, such as the genus Bacillus, phosphorus is often present at levels 2-3 orders of magnitude lower than those of other required ions (Ozanne, 1980).

Under phosphate limitation, organisms have evolved a number of adaptive strategies. These mechanisms include utilizing phosphate more efficiently in order to maintain essential metabolic pathways, and in times of plenty, storing excess phosphate in vacuoles so that it may be used to replenish the cytoplasmic pool as required. One example is Bacillus, where teichoic acid, a phosphate-rich polymer, is synthesized under phosphate-rich conditions but is replaced by teichuronic acid, lacking phosphate, under phosphate limitation (Hulett, 1996).

The phosphate limitation responses have been investigated extensively in plant and yeast cells. A set of enzymes that are involved in the metabolism of phosphate, including acid and alkaline phosphatases, are produced in yeast cells. Most of these enzymes are synthesized in response to the presence or absence of inorganic phosphate. The genes that encode these enzymes have been studied, one example being the PHO system of Saccharomyces. This system constitutes a complex family of structural genes coding for several phosphatases with different cellular localizations as well as permeases responsible for phosphate uptake. Expression of these genes is ultimately regulated by the simple effector, inorganic phosphate (Oshima, 1982), and excess phosphate stops the expression of these genes. A large set of regulatory genes control the synthesis of these enzymes, some of them act positively, others negatively (Vogel and Hinnen, 1990). Several reports also indicate a similar relationship between such phosphatase activities and phosphate limitation in other organisms, such as Achlya (Hill and Mullins, 1979), Thiobacillus (Seeger and Jerez, 1993), Tetrahymena (Rasmussen et al., 1992), E. coli (Scholten et al., 1995), Bacillus (Vinter et al., 1987; Jensen et al., 1993), and Penicillium (Salt and Gander, 1988).

The phosphatase activities are also known to increase in plants experiencing phosphate limitation (Goldstein et al., 1988; Lefebvre et al., 1990; Duff et al., 1991a, b), and the genetic basis for this relationship is also known (Sadka et al., 1994; Malboobi and Lefebvre, 1995).

Hydrolytic enzymes for carbohydrate polymers are repressed when microorganisms are growing in a rapidly utilizable carbon source. In Neurospora, glucose represses the synthesis of these enzymes, but without exogenous carbon, derepression takes place and the enzymes are produced (Rey et al., 1979). This kind of catabolite repression in the synthesis of β-glucanases has been observed in other fungi including several species of Penicillium (Stantos et al., 1977; 1978), Basidiomycete QM 806 (Friebe and Halldorf, 1975; Kitamoto et al., 1987), and Sclerotium (Rapp, 1989). Some bacterial species share a similar response (Lilley and Ball, 1974; Rosa et al., 1984). In the study on Penicillium, catabolite repression of β-glucanase synthesis was shown to be exerted at a pretranslational level, and glucose starvation gave an accumulation of the mRNA specific to this enzyme which was then translated (Stantos et al., 1978).

As expected, catabolite sugar repression is a widespread phenomenon in microorganisms (Saier, 1989; Gancedo, 1992), and cells grown on one type of sugar repress the expression of genes coding for enzymes that are required for the metabolism of other carbon sources. In plants and microorganisms sugars not only function as substrates for growth, but also affect sugar-sensing systems that initiate changes in gene expression. Both abundance and depletion of carbohydrates can enhance or repress expression of genes. In microbes, carbohydrate signals to sugar-responsive genes provide a method for these organisms to adjust to changes in the availability of essential nutrients. This capacity is vital to their survival and/or effective competition. In general, carbohydrate depletion enhances

expression of genes for reserve mobilization and export processes, whereas abundant carbon resources favor genes for storage and utilization (Koch, 1996).

Interactive effects between carbon and phosphate have been reported in plants and fungi. In A. bisexualis, the accumulation of total reserve β-glucans decreases by 50% under low phosphate concentration (Lee and Mullins, 1994). In suspension-cultured Catharanthus cells, the rate of carbon uptake and incorporation of smaller sugars into larger ethanol-soluble fractions are lower in phosphate-deficient medium. This result suggests that phosphate strongly affects the utilization of sugars by cultured plants cells through both the stimulation of sugar transport and the activation of metabolism (Li and Ashihara, 1989; 1990).

Fungal β-glucans and Associated β-(1->3)-glucanases

Glucans are the most abundant polysaccharides in fungi and are widely distributed. They can be divided into two groups, α- and β-glucans, and the most important group in fungi is the β-form, which includes both homopolysaccharides and heteropolysaccharides (Seviour et al., 1992).

Six different types of β-glucans have been described: linear β-(1->3)-glucans; β-(1->3)-glucans with occasional β-(1->6) branching, with or without phosphate; β-(1->3)-glucans with significant amounts of β-(1->6) linkages; β-glucans containing mostly β-(1->6) linkage; β-glucans containing β-(1->3), β-(1->4) and β-(1->6) linkages (Ruiz-Herrera, 1991).

β-Glucans are getting more attention because of their potential application in chemical, pharmaceutical and food industries. The β-(1->3)-glucans that have β-glucopyranosyl units attached by 1->6 linkages as single unit branching enhance the immune system systemically. This enhancement results in antitumor, antibacterial, antiviral, anticoagulatory and wound healing activities. The β-(1->3)-glucan backbone is essential for such enhancement, the most active polymers have degrees of branching between 0.2 and 0.33 (Bohn and Bemiller, 1995).

β-(1->3)-Glucans are found as cell wall components, and intra- or extracellular reserve materials (Faro, 1972; Coulter and Aronson, 1977; Cottingham and Mullins, 1985; Katohda et al., 1984; Rapp, 1989; Lee and Mullins, 1994; Mazur et al., 1995).

β-Glucans which accumulate intracellularly may be used as reserve material at critical stages of reproductive development (Wang and Bartnicki-Garcia, 1980; Lee and Mullins, 1994). Some β-glucans are secreted in the form of slimy material which may protect the cells from dessication and other harmful environmental conditions (Ruiz-Herrera, 1991). In the case of pathogenic fungi, β-glucans are important in cellular recognition, and in eliciting defense responses of infected plants (Ryan, 1987; Dixon and Lamb, 1990; Cote and Hahn, 1994). The most general role of β-glucans is a structural one, as the major component of fungal cell walls. Inhibition of β-glucan synthesis in yeast leads to cell lysis, and often death which results from a weakening of the cell wall (Perez et al.,1983; Miyata et al., 1985). These inhibitors are used as important antifungal compounds against both plant and animal pathogens.

Synthesis of β-(1->3)-glucan is beginning to be understood. A membrane-bound GTP-stimulated β-(1->3)-glucan synthase was dissociated into two soluble fractions, both

required for activity (Mol et al., 1994). One fraction is a GTP-binding protein which not only regulates glucan synthase but can be regulated in turn, constituting a link between cell cycle controls and wall morphogenesis. The other fraction is an integral membrane protein which contains the catalytic center that incorporates glucose from UDPG into the developing polymer in a vectorial manner.

Of the reported fungal β-(1->3)-glucans, some are phosphorylated (Wang and Bartnicki-Garcia, 1973; Lee and Mullins, 1994; Lee et al., 1996). In Phytophthora, Wang and Bartnicki-Garcia(1973) reported a phosphorylated β-(1->3)-glucan in sporangia, zoospores and cysts. This phosphorylated β-(1->3)-glucan contains one or two phosphate residues as monoester linkages at the C-6 hydroxyl groups of some glucose units. In Achlya, a phosphorylated cytoplasmic β-(1->3)-glucan has been recently isolated and characterized (Lee and Mullins, 1994; Lee et al., 1996). It contains 5% phosphate (w/w), and has both mono- and di-phosphoester linkages. Although the biological role of the reserve β-(1->3)-glucans is most often suggested as a source of energy or carbon or both, the biological role of the phosphate has not been well defined.

Many fungi synthesize extracellular, intracellular or cell wall-associated β-(1->3)-glucanases (Fleet and Phaff, 1975; Villa et al., 1978; Del Rey et al, 1979; Rey et al.,1979; Reichlet et al., 1981; Rey et al., 1982; Hien and Fleet, 1983; Rapp,1989; Muthukumar et al., 1993; Mrsa et al., 1993; Stahmann et al., 1993; Kanzawa et al., 1994; Pitson et al., 1995; Fontaine et al., 1997), and some detailed characterization has been accomplished (Bielecki and Galas, 1991; Mishra and Robbins, 1995).

Like the β-(1->3)-glucans, these β-(1->3)-glucanases have various biological functions. They are largely involved in fungal morphogenesis, including bud initiation, cell expansion, cell elongation, cell wall formation, cell conjugation and sporulation (Kamada et al., 1985; Cabib et al., 1988; Wessels, 1988; Fontaine et al., 1997). They can also be used as tools to determine the specific polysaccharide structures of the wide array of glucose polymers found in nature in order to better understand the relationship between structure and function, or to determine the effects of modifying their structures (Bielecki and Galas, 1991; Mishra and Robbins, 1995).

The synthesis of the fungal β-(1->3)-glucanases can be regulated in both positive and negative directions by such control mechanisms as induction, feedback inhibition and catabolite repression. Generally, their synthesis is markedly repressed when they are growing in a rapidly metabolizable carbon source (Bielecki and Galas, 1991).

There are only a few references which consider the mechanism of action of β-(1->3)-glucanases on a molecular level (Bielecki and Galas, 1991). One proposed mechanism of catalysis suggests that a histidine residue is involved at the active site, and the glucosidic oxygen linking glucose residue 1 and 2 is protonized by the imidiazole group of this residue. After the attack of a water molecule at C-1 of the first glucose residue, the cleavage of the C-1-O bond occurs with a loss of the proton from the hydroxyl group of C-1, results in the releasing of α-glucose (Jeffcoat and Kirkwood, 1987).

Fungal Sporulation

When conditions are unsuitable for further growth, such as the exhaustion of nutrients, or in host/parasite interaction where the death of the host or alternatively, the host successfully resists further infection, the fungus must then find other sites suitable for growth. Alternatively the fungus can go into a dormant state via sporulation until favorable condition return (Carlile and Watkinson, 1994).

Sporulation results in a morphological form that contrasts with the uniformity of structure found among vegetative cells, and thus is important for fungal classification. Sporulation is normally initiated by nutrient limitation, although it is sensitive to environmental conditions such as temperature, light or humidity. The exhaustion of a key nutrient required by a fungus would end the vigorous vegetative growth, and sporulation is then initiated. In Achlya, carbon limitation is the key step for sporulation, but synchrony can be achieved by Ca^{2+}-induction (Griffin, 1966).

When Achlya mycelium is suspended in a Ca^{2+} medium, sporulation is initiated. Several clearly defined morphological events occur. These events, in order, are: (1) remodeling of the hyphal tips from a pencil shape to a rounded, blunt shape, 2-3 h after the initiation; (2) concentration of the hyphal cytoplasm at the hyphal tips, 3-4 h after initiation; (3) separation (sporangium formation) of the concentrated cytoplasm from the remaining hyphal contents by rapid cross-wall formation, after 4-5 h; (4) cleavage of the segregated cytoplasm into spore initials; (5) spore differentiation, 5-8 h; and finally (6) spore release.

(O' Day and Horgen, 1974). As the biflagellate spores are released, they encyst, forming at the exit pore of the sporangium a cluster of spore cysts. This process utilizes about 12 % of the mycelial dry weight.

Significant biochemical changes are associated with the morphological events of sporulation. Therefore, sporulation may provide a convenient system for the investigation of carbohydrate metabolism, the roles of the reserve materials and the associated hydrolytic enzymes. In yeast the extraspore reserve carbohydrates synthesized early in development are completely degraded prior to spore formation, suggesting they have a role as a source of energy and carbon for spore formation (Rothman and Cabib, 1969; Becker et al., 1982; Katohda et al., 1988; Rua et al., 1993). A two to three-fold increase of acid phosphatase activity during Ca^{2+}-induced sporulation was reported in Achlya (O' Day and Horgen, 1974), but no function for the increase was suggested.

Chapter 3
ACTIVITY OF THE ENZYMES PHOSPHODIESTERASE AND PHOSPHOMONOESTERASE: RESPONSE TO NUTRIENT LIMITATION

Introduction

Phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) are the enzymes that catalyze the hydrolysis of phosphate-containing compounds, releasing organic and inorganic phosphate for biosynthesis.

Most fungi accumulate reserve materials during vegetative growth, and these materials are generally utilized under starvation conditions, or to support reproductive cycles. Previous results from this lab using Achlya indicate that a major reserve in the mycelium is a β-glucan, consisting of a neutral form (20%) and a phosphorylated form (80%) (Lee et al., 1996). Under conditions of starvation (no glucose in the culture medium) or Ca^{2+}-induced sporulation, the mycelial glucans decrease dramatically, and their total phosphate content decreases 49% (Lee and Mullins, 1994). Cottingham and Mullins (1985) reported a similar decrease in total mycelial glucan during asexual reproduction of Achlya.

Under phosphate limitation, organisms have evolved a number of adaptive strategies to cope with such growth limitation. These mechanisms include utilizing phosphate more

efficiently in order to maintain essential metabolic pathways, and in times of plenty, storing excess phosphate in vacuoles so that it may be used to replenish the cytoplasmic pool as required. In the case of Achlya, reserve materials have to be utilized, and thus PDEase and PMEase could be increased or activated for this function. Several reports indicate a relationship between such phosphatase activity and phosphate limitation. In Achlya, acid phosphatase activity doubles in a glucose-only medium when compared with that of the defined liquid medium containing 1.5 mM phosphate (Hill and Mullins, 1979). Similar findings are reported in Thiobacillus (Seeger and Jerez, 1993), and in Tetrahymena (Rasmussen et al., 1992). The induction of alkaline phosphatase synthesis under low phosphate condition is documented in both E. coli (Scholten et al., 1995) and Bacillus (Vinter et al., 1987; Jensen et al., 1993).

High total activity and high specific activity are essential for the final purification of these enzymes, thus, five different cultural conditions were tested for their ability to influence the production of PDEase and PMEase.

Materials and Methods

Culture of Organism

Achlya bisexualis Coker and A.Couch (ATCC#14524) was used in this study. The spore inoculum for liquid cultures was prepared according to the method of Thomas (1989). In this method, mycelium was first grown on corn meal agar (CMA), then plugs were removed from the edge of an expanding colony. Ten plugs were placed into 100 ml peptone-yeast extract-glucose (PYG) medium in a 250 ml flask for 16-18 h at 25 C, on a shaker run at 110 rpm. The medium was decanted and replaced with fresh 100 ml 0.5 mM $CaCl_2$ sporulation medium and the flask was returned to the shaker, after 1 and 2 h, this step was repeated. The flask was shaken for another 6 h, and then aliquots of the resulting spore suspension were used to inoculate liquid cultures.

Five different cultural conditions were tested for activities of PDEase and PMEase. The first was modified enriched medium (MEM) (Cottingham and Mullins, 1985) (Table 1), 200 ml with 10 ml of the above spore suspension in a 500 ml Erlenmeyer flask. Growth was at 25 C and 110 rpm. The mycelium was harvested at 36, 48, 60, 72, 84 and 96 h. MEM is the standard against which other media will be compared, and it contains 1.5 mM KH_2PO_4. The second was MEM-minus-KH_2PO_4 (MEM-minus-P), following growth in 200 ml MEM for 36 h, the mycelium was washed with sterilized water before being transferred to 200 ml fresh MEM-minus-P, and then harvested at 0, 12, 24, 36, 48, 60 and 72 h. The third was different phosphate concentrations of 0.015, 0.03, 0.15 and 1.5 mM in 200 ml MEM. The mycelium was harvested at 60 h. The fourth was a time course using the lowest phosphate concentration (0.015 mM) in 200 ml MEM and with harvest at 36, 48, 60, 72, 84 and 96 h.

The fifth was Ca^{2+} -induced sporulation, following growth in 200 ml MEM for 24 h, the mycelium was transferred to 0.5 mM $CaCl_2$ for periods of 0, 3, 5, 8 and 12 h.

Extraction of Enzymes

Triplicate flasks from each of the five cultural conditions were harvested and extracted separately. The mycelium was poured into a Buchner funnel containing a nylon filter and washed with a large volume of distilled water. The initial extraction followed that of Cottingham and Mullins (1985). In this procedure, washed mycelium is homogenized in 0.3 M Tris-HCl buffer, pH 7.6 containing 0.2 mM EDTA, then filtered through nylon and centrifuged at 1500 g for 10 min to remove cellular debris. The supernatant is then centrifuged at 12,000 g for 20 min and the resulting pellet discarded. The clarified supernatant is defined as crude enzyme.

Assay of Enzyme Activity

The standard enzyme assay consisted of 0.4 mL substrate and 0.1 mL enzyme solution. The reaction mixture was incubated at 37 C for a suitable time (20 to 60 min). The reaction was stopped by adding 2 mL 0.2 M Na_2CO_3, and the product (p-nitrophenol) was measured at 410 nm and related to a standard curve of p-nitrophenol.

Substrate was prepared by modifying the method of Hill and Mullins (1979), 2 mM p-nitrophenyl phosphate (for PMEase) or 2 mM bis(p-nitrophenyl) phosphate (for PDEase), plus 2 µmole $MgCl_2$ were dissolved in 50 mM Tris-HCl buffer, pH 7.6. One unit of enzyme activity is defined as the liberation of 1 µmol p-nitrophenol min^{-1}. The units are given on the basis of 1 mL of enzyme solution.

The enzyme activity of the buffer fraction from Cibacron blue chromatography was also assayed using the phosphoglucan of Achlya. The reaction mixture contained 0.4 ml of 1 mg mL^{-1} phosphoglucan in 50 mM Tris-HCl buffer, pH 7.6 and 0.1 mL enzyme solution. After 48 h at 37 C, the inorganic phosphate was measured by the Ames method (1966).

Protein content in the enzyme solution was estimated by the method of Lowry et al., (1951) with bovine serum albumin as the standard. Glucose in the culture medium was measured by the glucose oxidase-peroxidase method (Gascon et al., 1968) with glucose as the standard.

Table 1. The composition of modified enriched medium (MEM) (Cottingham and Mullins, 1985)

Monosodium-L-glutamate	3.0 mM
D-Glucose	11.1 mM
Tris (hydroxymethyl) aminomethane	10.0 mM
Casein hydrolysate (enzymatic)	0.015% (w/v)
L-Methionine	0.1 mM
KCl	2.0 mM
$MgSO_4$	0.5 mM
$CaCl_2$	0.5 mM
HEDTA	72.0 μM
KH_2PO_4	1.5 mM
$Fe(NH_4)_2(SO_4)_2$	36.0 μM
$ZnSO_4$	15.0 μM
$MnSO_4$	15.0 μM
Sulfosalicylic acid	46.0 μM

Results

Mycelial Total and Estimated Specific Activities of PDEase and PMEase from Standard MEM Culture.

MEM medium contains 1.5 mM phosphate and was developed in this lab to culture Achlya. The total activity flask^{-1} for PMEase increased from 36 to 72 h, then decreased (Fig. 1). The specific activity increased up to 84 h (Fig. 2). The specific activity of PMEase was higher at 84 h and 96 h than at 72 h, probably because of a lower protein concentration in the enzyme solution at 84 and 96 h. Both total and specific activity of PDEase increased rapidly with time and peaked at 72 h (Figs. 1 - 2). Mycelial fresh weight and protein in the crude enzyme solution decreased from 36 to 96 h (data not shown).

Mycelial Total and Estimated Specific Activities of PDEase and PMEase from MEM-minus-Pi Culture.

Previous results showed that when mycelium was cultured on MEM, no glucose remained in the medium after 36 h (Cottingham and Mullins,1985). When such mycelium is transferred to fresh MEM minus phosphate, the total and specific activities of PMEase increased and reached a peak after 36 h (Figs. 3 - 4). Total activity of PDEase increased and peaked at 60 h, while specific activity increased up to 72 h. (Figs. 3 - 4).

Mycelial Total and Estimated Specific Activities of PDEase and PMEase Produced Under Different Phosphate Concentrations.

When mycelium was cultured on MEM with different phosphate concentrations, the results showed that PDEase and PMEase activities decreased with an increase in the phosphate concentration, except for total PMEase activity (Figs. 5 - 6). The fresh weight of the mycelium increased with an increase in phosphate concentration (data not shown).

Mycelial Total and Estimated Specific Activities of PDEase and PMEase from The Lowest Phosphate Culture.

The previous experiment showed that mycelium cultured on MEM with 0.015 mM phosphate had the highest total and specific activities for both PDEase and PMEase. Thus, this medium was selected for a growth experiment. The total and specific activities of PMEase decreased during growth over 36 - 96 h (Figs. 7 - 8). Total activities of PDEase increased during the period of 36 - 60 h and then declined (Fig. 7). Specific activity of PDEase increased during the same period and then remained high (Fig. 8).

Mycelial Total and Estimated Specific Activities of PDEase and PMEase During Ca^{2+} -induced Sporulation.

Total and specific activities of PMEase increased sharply in the early stages of sporulation, then steadily decreased (Figs. 9 - 10). The total and specific activities of PDEase increased throughout sporulation (Figs. 9 - 10).

The highest total and specific activities of PDEase and PMEase from each of the different cultural conditions are compared in Table 2.

Fig. 1. Total mycelial activities of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) of A. bisexualis on modified enriched medium (MEM). Enzyme was extracted from the mycelium with 0.3 M Tris-HCl buffer, pH 7.6 containing 0.2 mM EDTA. p-Nitrophenyl phosphate was the substrate for PMEase, bis (p-nitrophenyl) phosphate for PDEase. The assay consisted of 0.4 ml 2 mM substrate in 50 mM Tris-HCl buffer, pH 7.6 and 0.1 ml enzyme solution at 37 C. p-Nitrophenol was measured at 410 nm and related to a standard p-nitrophenol curve. One unit of activity is defined as the liberation of 1 μmol p-nitrophenol min^{-1}. The units are given on the basis of 1 mL of enzyme solution. Each point is the average of three measurements. Bars represent standard deviation.

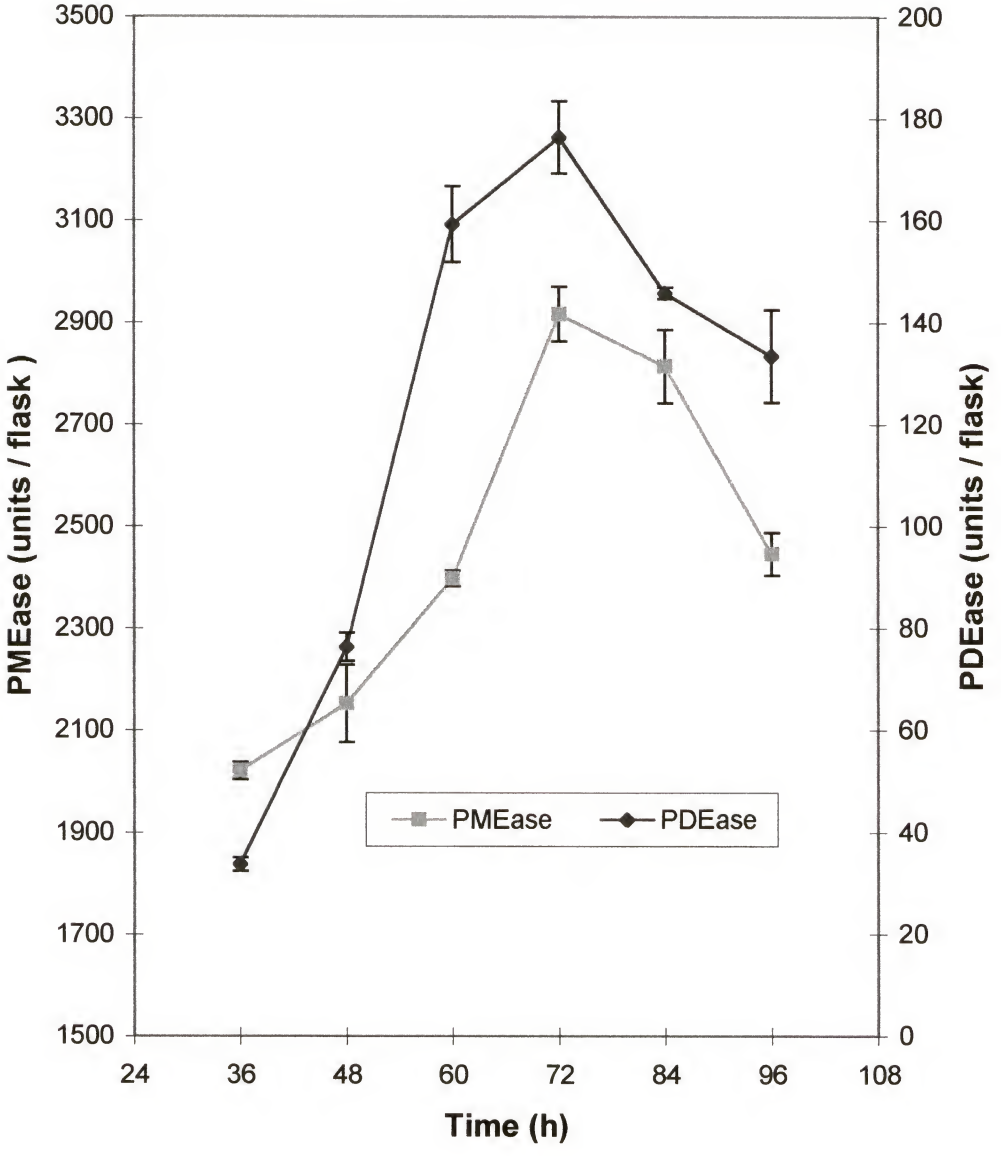

3500
3300
3100
2900
2700
2500
2300
2100
1900
1700
1500
PMEase (units / flask)
200
180
160
140
120
100
80
60
40
20
0
PDEase (units / flask)
24
36
48
60
72
84
96
108
Time (h)
PMEase
PDEase

Fig. 2. Estimated specific activities of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) of A. bisexualis. Experimental methods were the same as Fig. 1. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

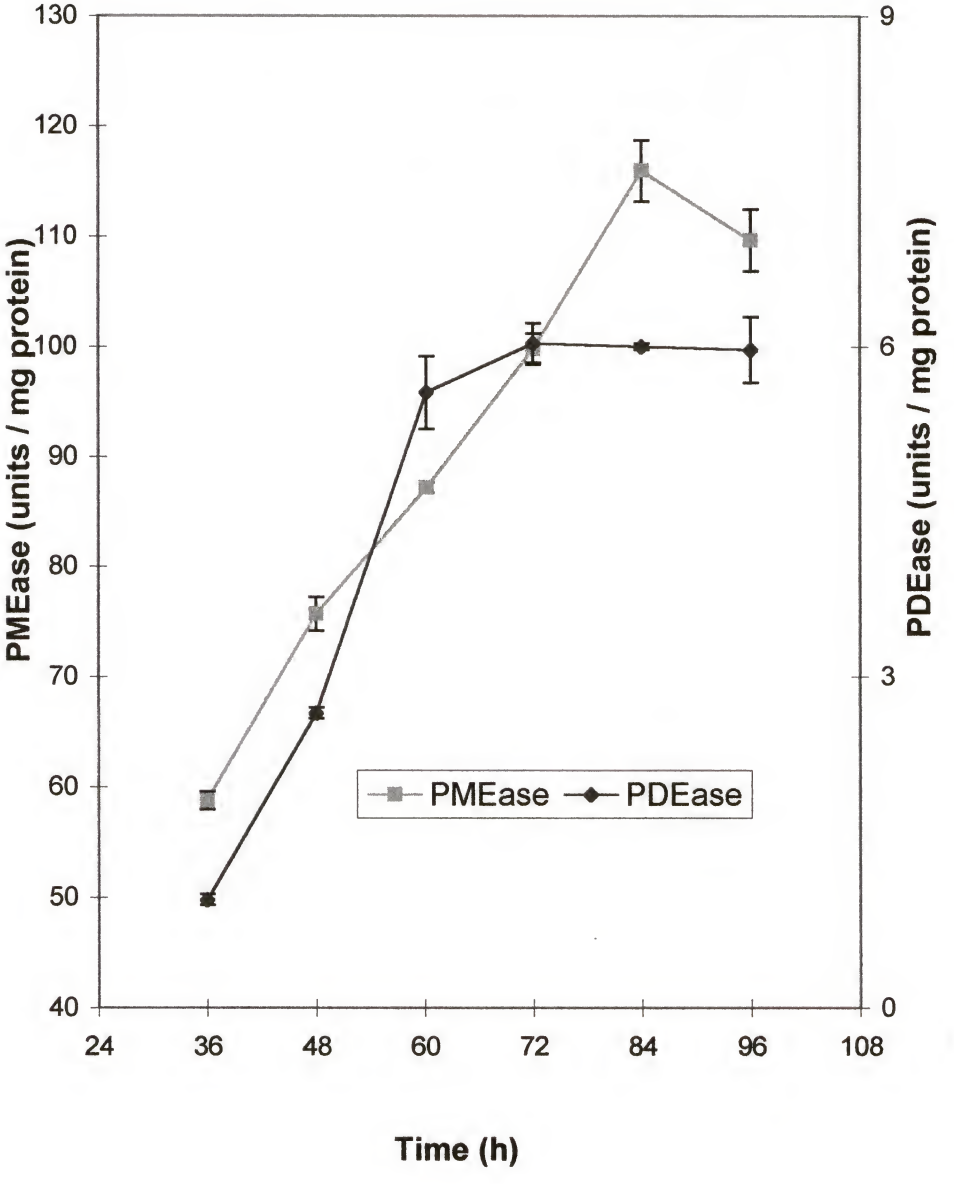

130
120
110
100
90
80
70
60
50
40
9
6
3
0
24
36
48
60
72
84
96
108
PMEase (units / mg protein)
PDEase (units / mg protein)
Time (h)
PMEase
PDEase

Fig. 3. Total mycelial activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A. bisexualis. Modified enriched medium (MEM) supported the growth for 36 h, then the mycelium was transferred to fresh MEM without phosphate and growth continued from 0 to 72 h. Enzyme extraction, assays and units were the same as Fig. 1.

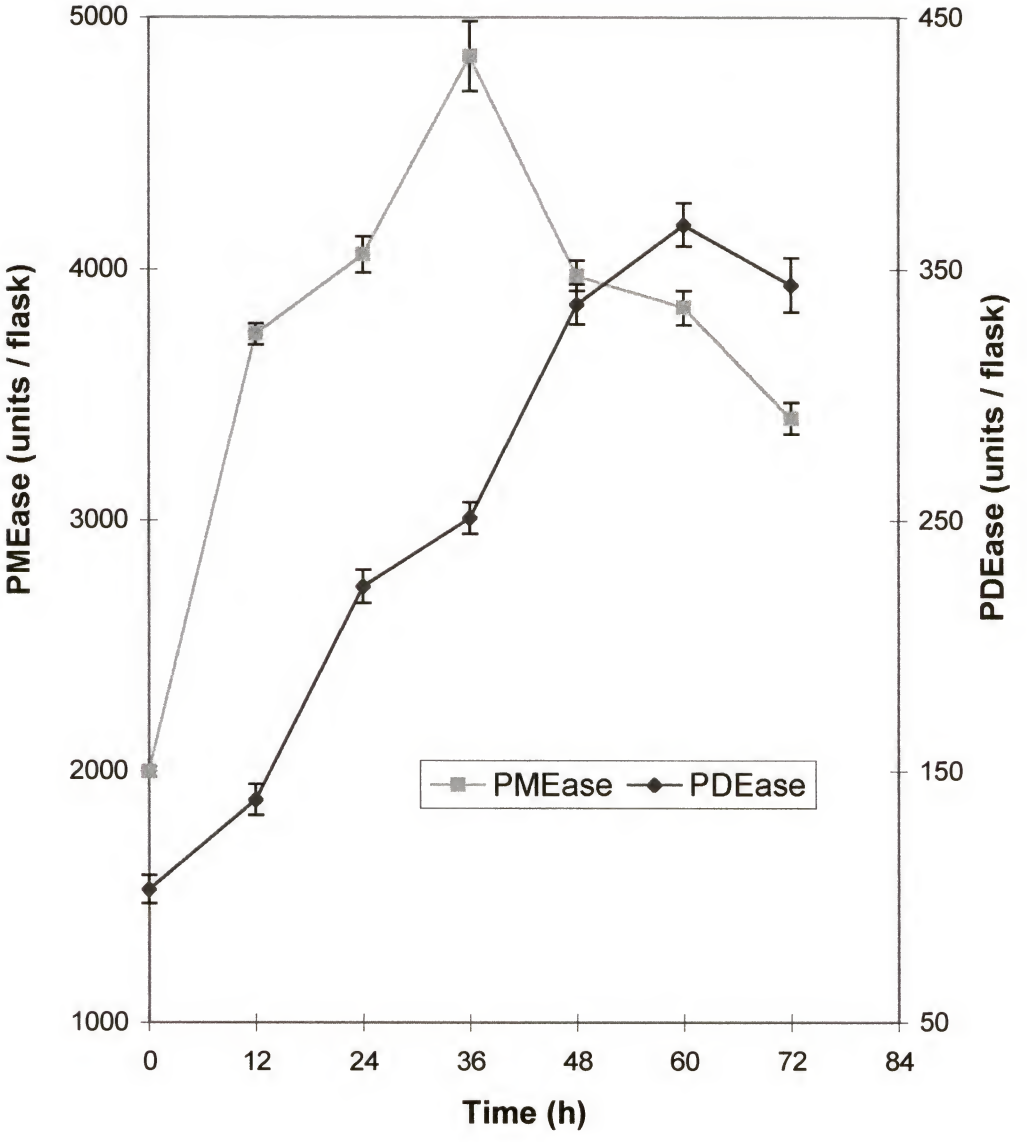

5000
4000
3000
2000
1000
450
350
250
150
50
PMEase (units / flask)
PDEase (units / flask)
0
12
24
36
48
60
72
84
Time (h)
PMEase
PDEase

Fig. 4. Estimated specific activities of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase)of A. bisexualis. Experimental methods were the same as Fig. 3. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

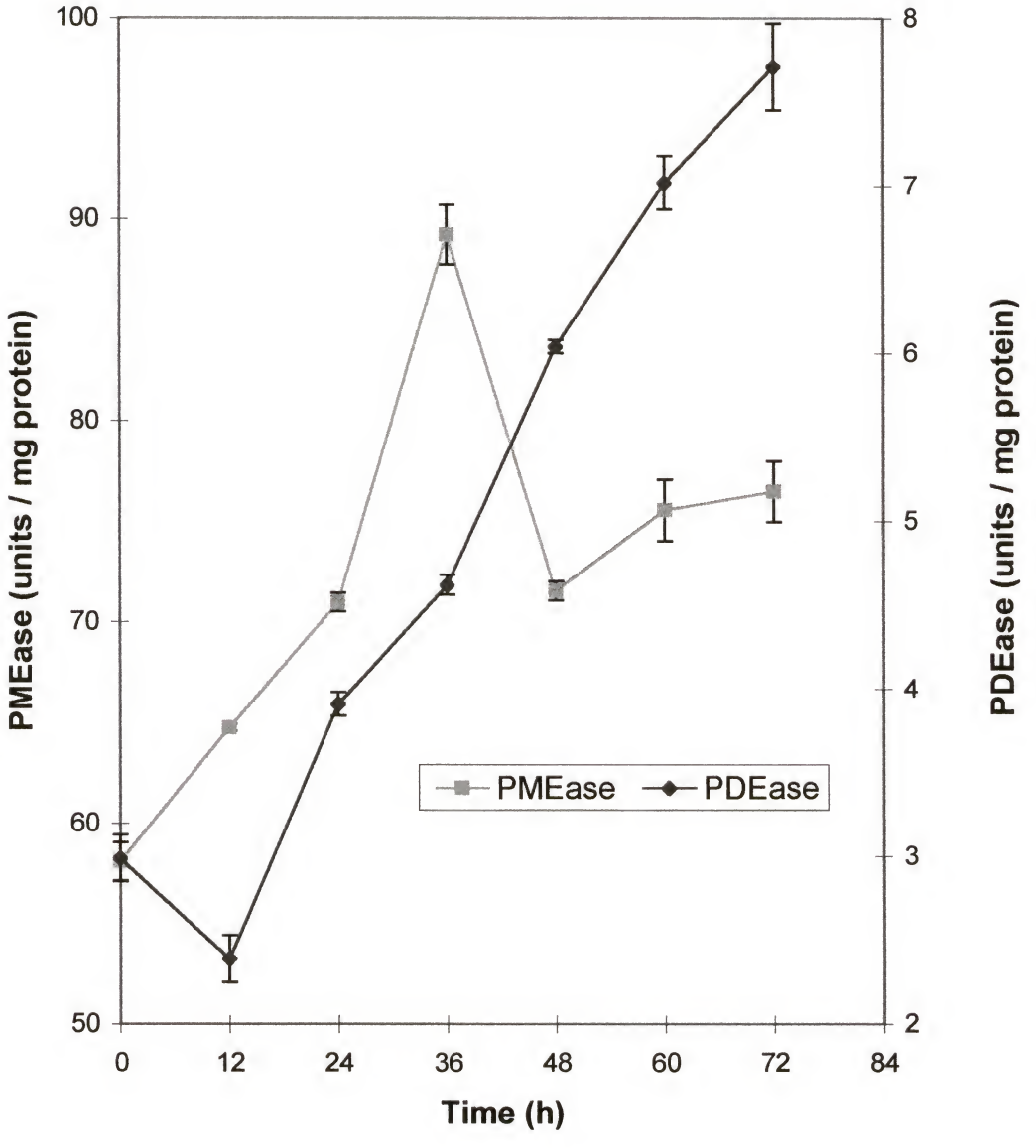

100
90
80
70
60
50
8
7
6
5
4
3
2
0
12
24
36
48
60
72
84
PMEase (units / mg protein)
PDEase (units / mg protein)
Time (h)
PMEase
PDEase

Fig. 5. Total mycelial activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A. bisexualis. Modified enriched medium with phosphate at 0.015, 0.03, 0.15 and 1.5 mM supported the growth for 60 h. Enzyme extraction, assays and units were the same as Fig. 1.

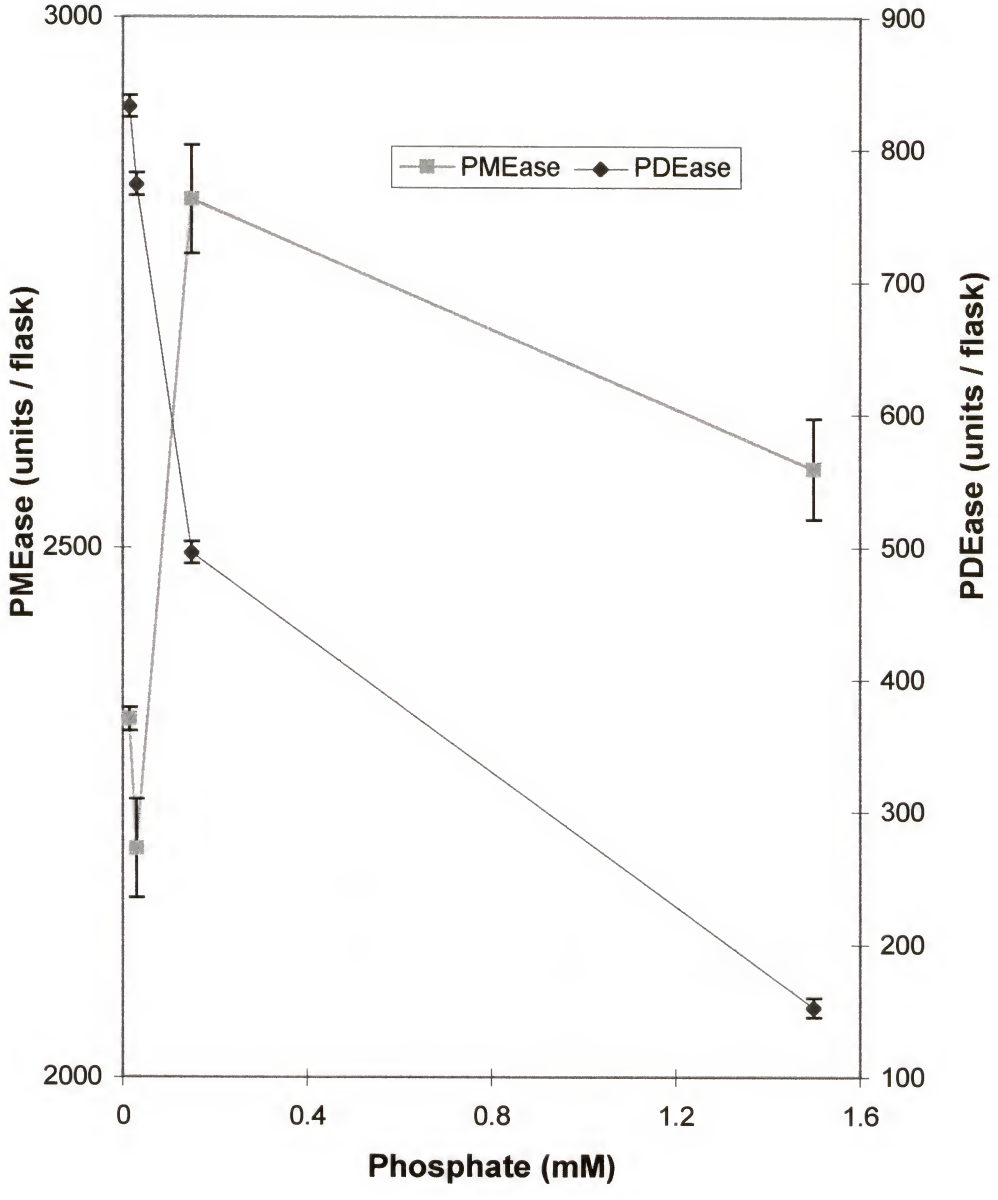

3000
2500
2000
900
800
700
600
500
400
300
200
100
PMEase
PDEase
PMEase (units / flask)
PDEase (units / flask)
0
0.4
0.8
1.2
1.6
Phosphate (mM)

Fig. 6. Estimated specific activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A. bisexualis. Experimental methods were the same as Fig. 5. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

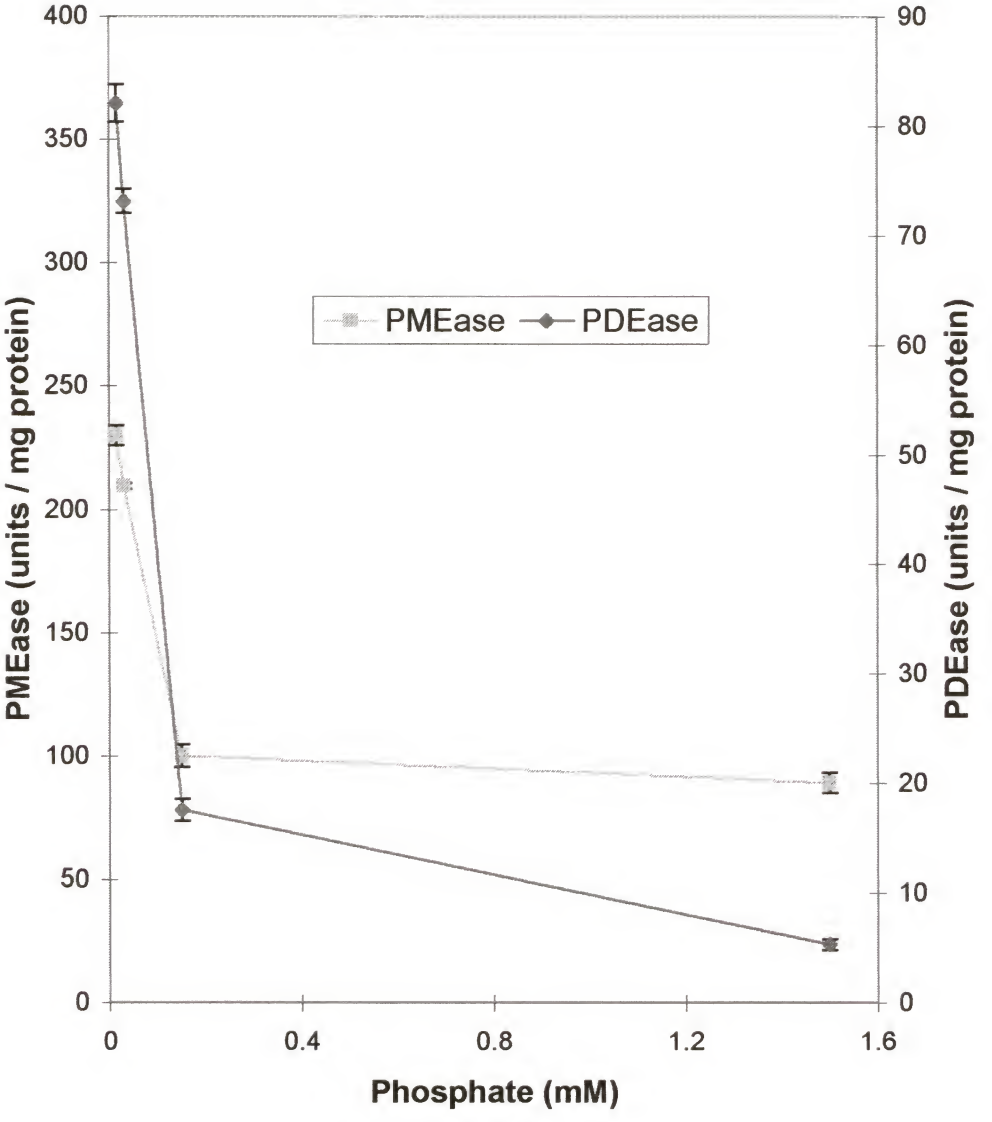

PMEase
PDEase
PMEase (units / mg protein)
PDEase (units / mg protein)
Phosphate (mM)
400
350
300
250
200
150
100
50
0
90
80
70
60
50
40
30
20
10
0
0
0.4
0.8
1.2
1.6

Fig. 7. Total mycelial activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A bisexualis on modified enriched medium with phosphate at 0.015 mM. Enzyme extraction, assays and units were the same as Fig. 1.

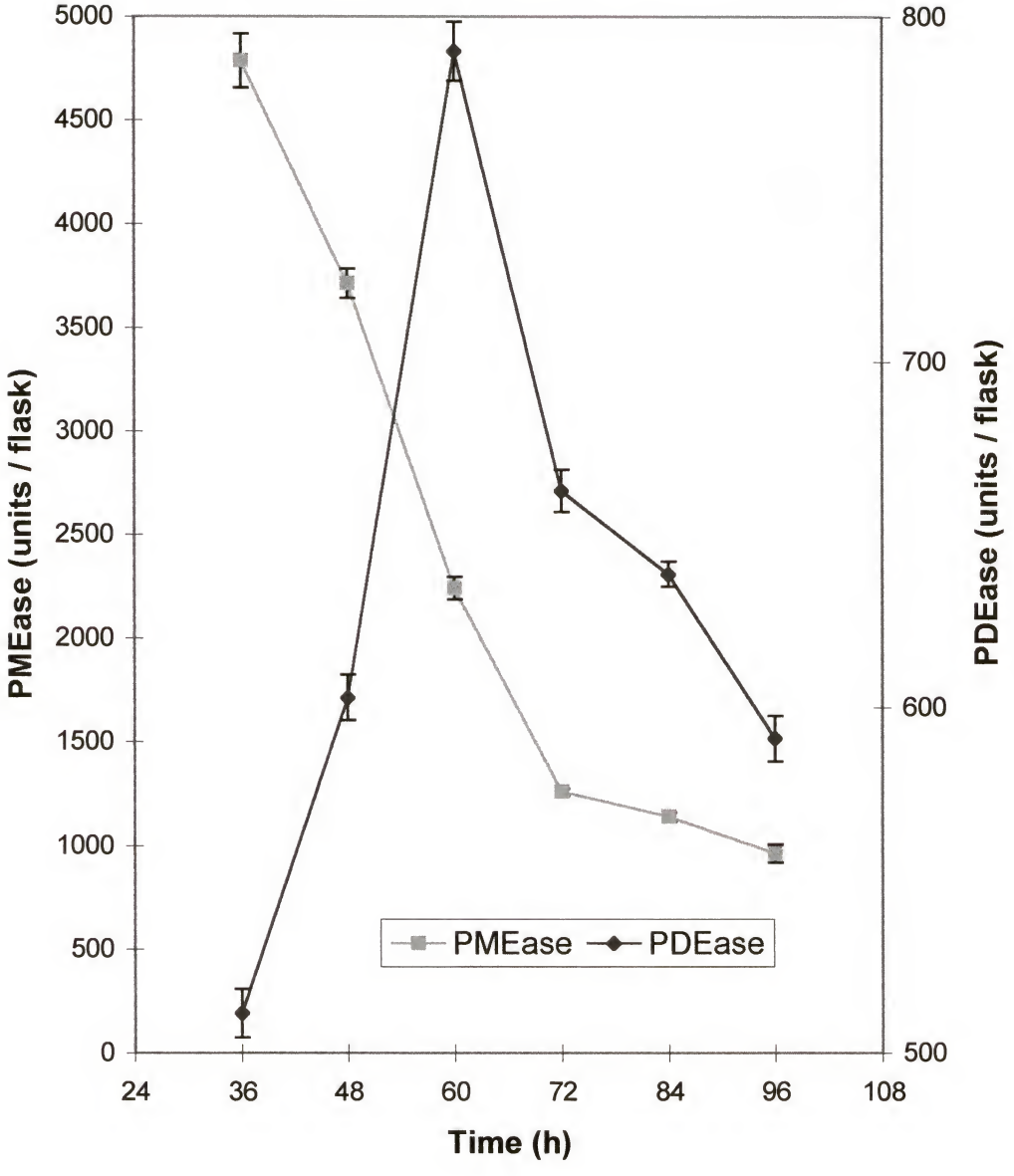

5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
PMEase (units / flask)
800
700
600
500
PDEase (units / flask)
24
36
48
60
72
84
96
108
Time (h)
PMEase
PDEase

Fig. 8. Estimated specific activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A bisexualis. Experimental methods were the same as Fig. 7. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

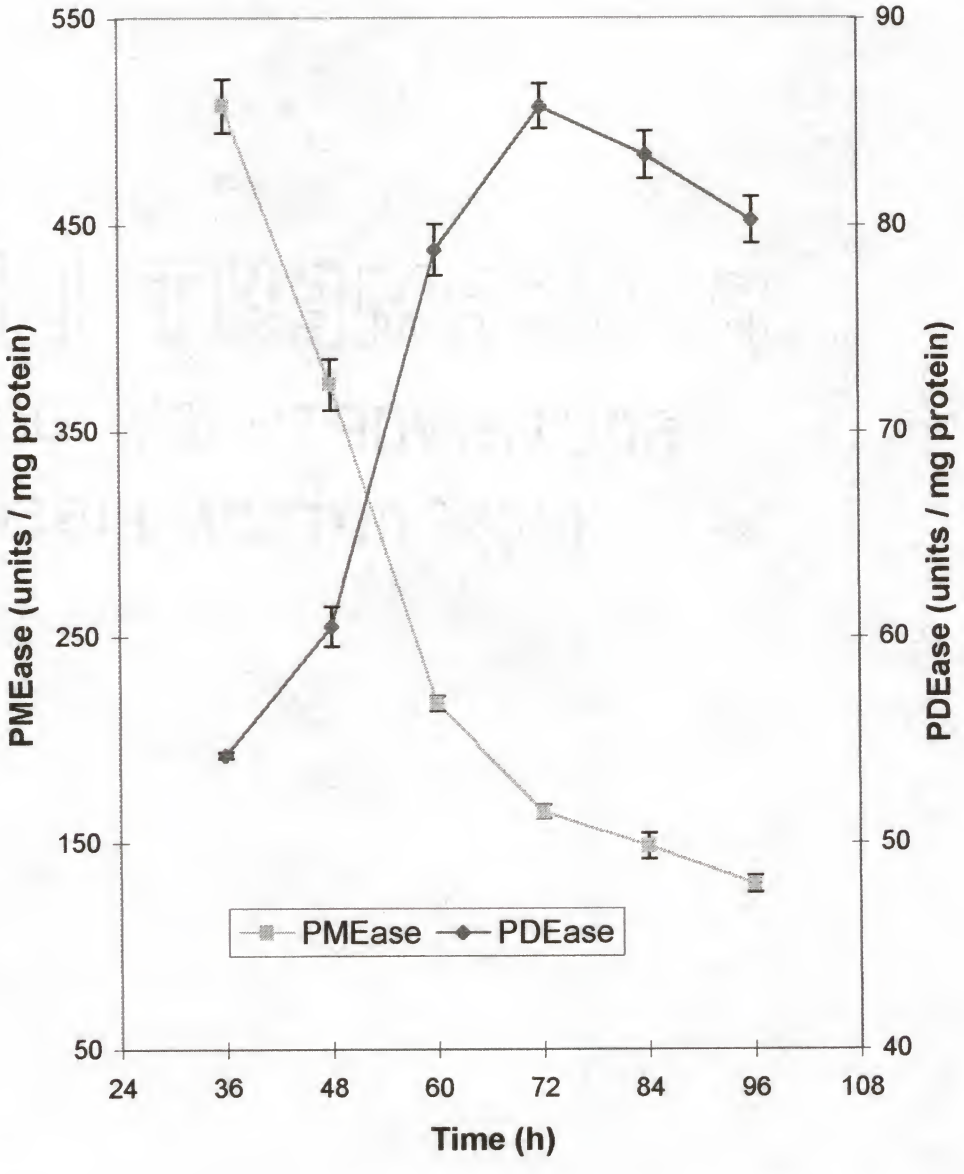

550
450
350
250
150
50
90
80
70
60
50
40
PMEase (units / mg protein)
PDEase (units / mg protein)
PMEase
PDEase
24
36
48
60
72
84
96
108
Time (h)

Fig. 9. Total mycelial activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A. bisexualis during Ca^{2+}-induced sporulation. Modified enriched medium supported the growth for 24 h, then sporulation was initiated by Ca^{2+} medium. Enzyme extraction, assays and units were the same as Fig. 1.

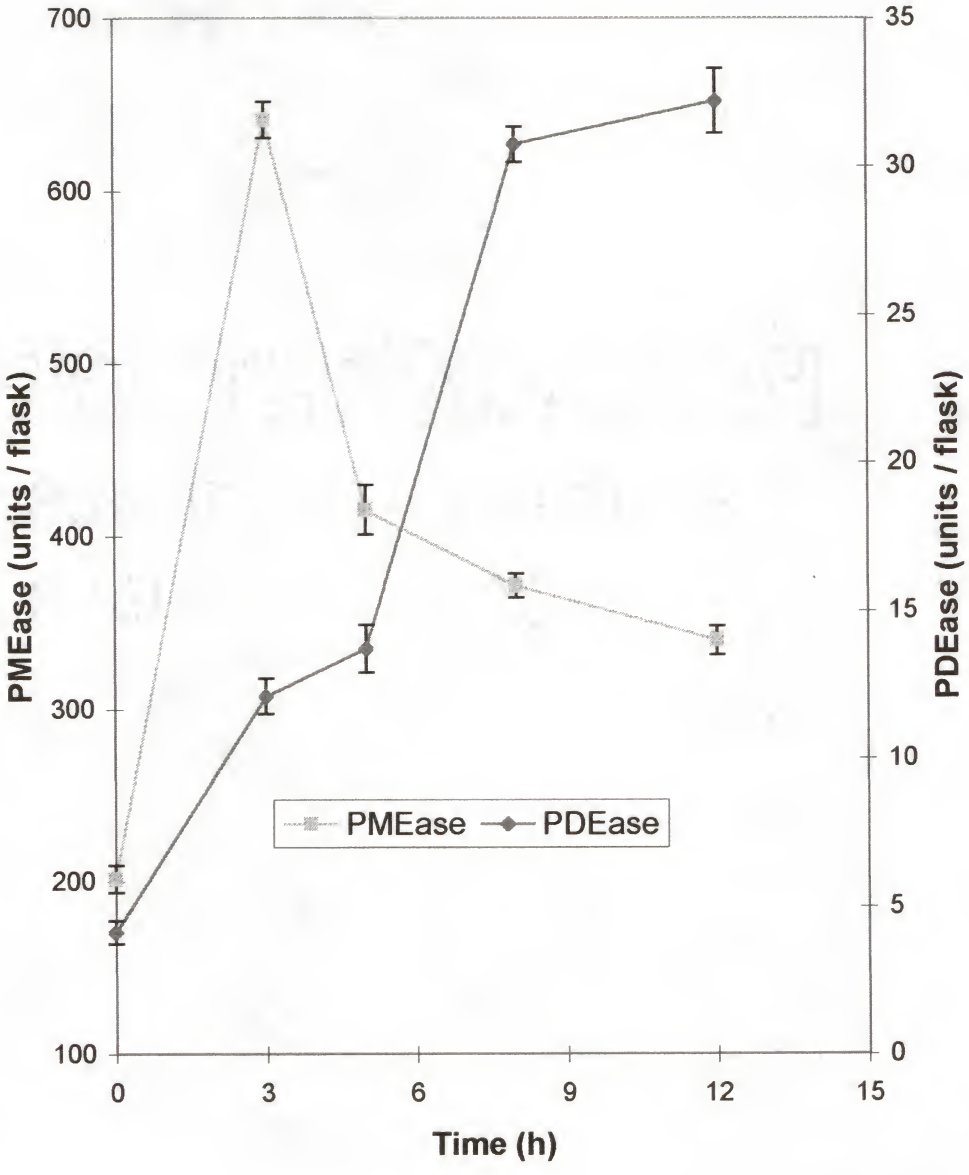

700
600
500
400
300
200
100
PMEase (units / flask)
35
30
25
20
15
10
5
0
PDEase (units / flask)
0
3
6
9
12
15
Time (h)
PMEase
PDEase

Fig. 10. Estimated specific activities of phosphodiesterase (PDEase) and phosphomonoesterase (PMEase) of A. bisexualis. Experimental methods were the same as Fig. 9. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

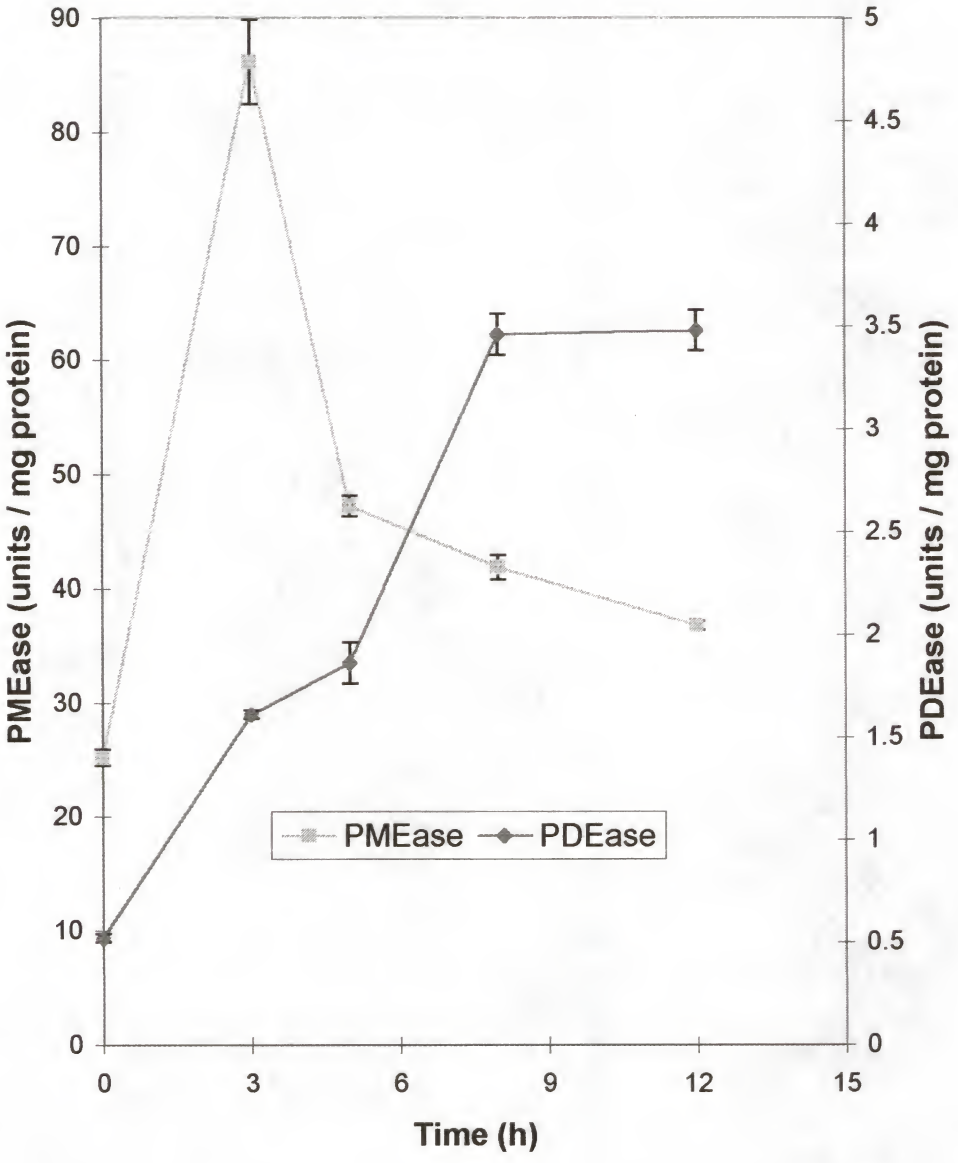

PMEase (units / mg protein)
PDEase (units / mg protein)
Time (h)
PMEase
PDEase
0
10
20
30
40
50
60
70
80
90
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
3
6
9
12
15

Table 2. The comparison of the highest total and specific activities of PDEase and PMEase of the different cultural conditions

Medium[a]	Total Activity (Units flask^{-1})				Specific Activity (Units mg^{-1} protein)			
	PDEase	Ratio[b]	PMEase	Ratio[b]	PDEase	Ratio[b]	PMEase	Ratio[b]
MEM	176	--	2917	--	6.03	--	116	--
MEM-P	368	2.09	4846	1.66	7.71	1.28	89.2	0.77
Low P	790	4.48	4786	1.64	85.8	14.2	507	4.38
Sporulation	32.2	0.18	641	0.22	3.48	0.58	86.2	0.74

[a] Media are: MEM = modified enriched medium; MEM-P = MEM minus phosphate; Low P = MEM with phosphate at 0.015 mM; Sporulation = after 24 h growth on MEM, mycelium was transferred to 0.5 mM $CaCl_2$ solution.

[b] The ratio as compared with MEM.

Discussion

PDEase and PMEase are the enzymes that hydrolyze phosphate from various phosphate-containing compounds. In order to increase the opportunity for their purification, five different cultural conditions were studied for their influence on PDEase and PMEase activities.

In the standard MEM medium, it was previously shown that after 36 h of culture, no glucose remained in the medium, and continued growth to 96 h utilized the cytoplasmic reserve glucans (Lee and Mullins, 1994). My results demonstrate increases in both PDEase and PMEase activities during the first 72 h of growth (Figs. 1 - 2). The total PMEase activity peaked at 72 h, and this represents an increase of 45%. The total PDEase activity peaked at 72 h and this represents an increase of 5.2-fold. These results suggest that when the mycelium is under external starvation conditions, both PDEase and PMEase activities increase, paralleling the decline in stored β-glucans. When the culture continued beyond 72 h, the mycelial growth slows and PDEase and PMEase activities decrease.

In the MEM-minus-P culture, the mycelium was first cultured on MEM for 36 h, a point at which no glucose remains in the culture medium, before being transferred to a fresh MEM without phosphate. Previous results indicated that when mycelium is cultured for 36 h in MEM, then supplemental glucose is added and growth continued for 24 h, the mycelial dry weight increases 58%, mycelial glucan increases 135%, but the phosphate content of this phosphoglucan is reduced by one-half (Lee and Mullins, 1994). These results suggest that when a supplemental carbon source is supplied, the mycelium responds in both growth and

accumulation of reserve materials. My intention for the MEM-minus-Pi culture condition is to create a limitation of phosphate while other nutrients are available, and thus to determine if the mycelium will utilize its phosphate reserve. This expectation was realized in a marked increase of PMEase activity, 2.4-fold for total activity and 54% for specific activity (Figs. 3 - 4). The PDEase activity increased up to 60 h (Figs. 3 - 4), total activity increased 3.6-fold and specific activity 2.6-fold. Thus, under phosphate limitation, both PDEase and PMEase are increased and could participate in the depolymeration of the β-glucans. In Penicillium, increases of PMEase and PDEase activities occur under phosphate limitation (Salt and Gander, 1988). Similar results are reported for other organisms (Smith, 1990; Goldman et al., 1990). Phosphate limitation may not only enhance phosphatase activity, but also increase the rate of phosphate uptake (Jennings, 1995). This increased influx of phosphate under phosphate-starved or phosphate-deficient cultural conditions is reported in several species of fungi and algae (Dick and Quinn, 1995; Goldstein , 1991).

In additional experiments, MEM with different phosphate concentrations was used to support growth for 60 h. The results show that both total and specific PDEase activities decrease with an increase of phosphate in the range of 0.015 -1.5 mM (Figs. 5 - 6). The highest activities are obtained with the lowest phosphate concentration. This highest total PDEase activity is 4.5-fold higher than that of the standard MEM medium and specific activity is 14.2-fold higher. Total PMEase activity is similar to that of the standard MEM medium, but specific activity is 2.6-fold higher. Previous results with mycelium cultured on MEM with 0.15 mM phosphate showed that growth is not markedly affected (dry weight decreases 11%), but the accumulation of β-glucans decreases by 50%. In addition, the ratio of the neutral and phosphoglucan is reversed and the phosphate content of the glucan is reduced

from 5% to 1 % (w/w) (Lee and Mullins, 1994). These results demonstrate that under 0.15 mM phosphate condition, although the mycelium can still maintain growth, the accumulation of β-glucan and the incorporation of phosphate into the glucan are significantly reduced. Current data confirm that in 0.15 mM phosphate, mycelial growth is similar to that of the standard MEM medium (data not shown), but PDEase and PMEase activities increase significantly. With lower phosphate (0.03 and 0.015 mM), mycelial growth is reduced and fresh weight is only 61% of that in the standard MEM medium (data not shown). The PDEase and PMEase activities continue to increase (Figs. 5 - 6), especially the specific activities (Fig. 6). This result suggests that PDEase and PMEase activities are sensitive to phosphate limitation. A similar result is reported in Acinetobacter, where the amount of biomass is proportional to the phosphate concentration in the medium, and the specific activities of alkaline phosphatase and the phosphate uptake system are induced when the phosphate concentration is below a certain value (Bonting et al., 1992).

The results from a growth kinetic experiment with the lowest phosphate (0.015 mM) medium show that the total and specific PMEase activities decrease constantly from 36 to 96 h (Figs. 7 - 8), indicating that an early peak may have been reached before 36 h. Both total and specific activities of PDEase increase significantly from 36 to 60 h, then decrease (Figs. 7 - 8).

Under Ca^{2+}-induced sporulation, the PMEase total activity increases 318% during the first 3 h and specific activity 342% (Figs. 9 - 10). The PDEase activities increase up to 12 h including both total and specific activities (Figs. 9 - 10). In Achlya, two to three-fold increase of acid phosphatase activity during Ca^{2+}-induced sporulation was reported (O' Day and Horgen, 1974), but no function for the increase was suggested. The relationships between

phosphatase activity and sporulation are revealed in several organisms. The increase of alkaline phosphatase activity is suggested to be linked with sporulation in Anabaena, and an increase in phosphate concentration delays spore initiation (Pandey et al., 1991). In Bacillus, a sporulation-induced alkaline phosphatase, which is independent of phosphate concentration, is reported (Warren, 1968; Glenn and Mandelstam, 1971). Recently, the genes that regulate this enzyme have been studied (Brookstein et al., 1990; Birkey et al., 1994). The results suggest multiple structural genes encoding alkaline phosphatase, which are induced either by phosphate starvation, sporulation, or both.

In summary, the highest PDEase activities, both total and specific, are observed with the lowest phosphate, and this highest specific activity is about 14.2-fold higher than that of the standard MEM medium. The highest total PMEase activity is obtained from the downshift from MEM to MEM-minus-P, mainly because of the larger mycelial mass, but the highest specific activity is obtained in the lowest phosphate, which is 4.4-fold higher than that of the standard MEM medium (Table 2). These results suggest that phosphate limitation exerts a major control on PDEase and PMEase activities in Achlya. Originally, the sporulation condition was thought to be the most effective way to promote the production of PDEase and PMEase, but actually the total mycelial activities of PMEase and PDEase are very low, because for the sporulation experiment, the mycelium was harvested at 24h, at which the mycelium has maximum amounts of well-grown tips that are the sites for sporangia, but the mycelium mass is small (data not shown).

My results also indicate that the PMEase activity peaks earlier than the PDEase. Since the major reserve phosphate source, the phosphoglucan, contains both monoester (80%) and diester linkages (20%), these results may suggest that under phosphate limitation, the

mycelium may utilize a PMEase to hydrolyze the monoester phosphate before activating a PDEase to hydrolyze the diester linkage, which makes available additional monoester linkages.

Chapter 4
ACTIVITY OF THE ENZYMES β-GLUCANASE AND β-GLUCOSIDASE: RESPONSE TO NUTRIENT LIMITATION

Introduction

β-(1->3)-Glucanases are enzymes capable of hydrolyzing β-(1->3)-glucans. They are frequently synthesized by a variety of microorganisms and plants, and are classified according to the composition and linkages of their substrates. They can be further classified as endo- and exo-types. Endoglucanases attack a glucan chain at a specific oligomer unit or randomly at any position except at or very near the termini, while exoglucanases are able to attack the glucan chain progressively from the nonreducing termini, releasing glucose (Rapp, 1989). Furthermore, endoglucanases act in such a way that their products retain the configuration of the substrates, whereas exoglucanases invert the configuration of the products (Reese, 1977). Endo-β-(1->3)-glucanases can be further defined as specific or non-specific, according to the substrate attacked and the linkage cleaved. The specific β-(1->3)-glucanase (EC 3.2.1.39) acts only on β-(1->3) linkages and not on mixed-linked β-(1->3/1->4)-glucans, such as lichenan and cereal β-glucans. The non-specific β-(1->3/1->4)-glucanase (EC

3.2.1.6) apparently binds to β-(1->3) linkages, but cleaves either β-(1->3) or (1->4) bonds. Assays with substrates having single or mixed linkages distinguish specific from non-specific forms (Mishra and Robbins, 1995).

β-Glucosidase (EC 3.2.1.21) catalyzes hydrolysis of the β-D-glucosidic linkage, and shows activity against a wide variety of substrates. It is universally distributed among many species of microorganisms and plants.

Most fungi accumulate cytoplasmic reserve materials when the growth medium contains an excess of reduced carbon (Pfyffer and Rast, 1980; Lee and Mullins, 1994), while some secrete carbohydrate metabolites into the medium (Halleck, 1967; Niederpruem et al., 1977; Rinaudo and Vingendon, 1982; Rapp, 1989). Among the cytoplasmic carbohydrates, one example is the (1->3)-β-glucan with (1->6)-β-linked side chains (Lee and Mullins, 1994; 1996).

Reserve materials accumulated during vegetative growth are generally utilized under starvation conditions, or to support reproductive cycles. Under conditions of starvation, defined here as no glucose in the culture medium, or Ca^{2+}-induced sporulation, the cytoplasmic glucans of Achlya bisexualis decrease dramatically (Lee and Mullins, 1994). Cottingham and Mullins (1985) report a similar decrease of mycelial glucan during asexual reproduction of A. ambisexualis.

The relationship between activities of β-glucanases and β-glucosidase and nutrient starvation or reproduction is well-documented for bacteria, fungi and plants. In A. ambisexualis, a four-fold increase of β-glucosidase activity under starvation conditions and a 2.5-fold increase of this activity under Ca^{2+}-induced sporulation are

reported (Cottingham and Mullins, 1985). In the fungus Sclerotium, significant amounts of extracellular and low levels of intracellular β-glucanase and β-glucosidase activities are produced under carbon starvation (Rapp, 1989). In yeast, high β-glucanase activity during sporulation is documented in many species (Fleet and Phaff, 1975; Villa et al., 1978; Del Rey et al, 1979; Rey et al.,1979; Reichlet et al., 1981; Rey et al., 1982; Hien and Fleet, 1983; Muthukumar et al., 1993). Significant amounts of β-glucanase are present under carbon starvation in Penicillium (Stantos et al., 1977; 1978), in several Basidiomycetes (Friebe and Halldorf, 1975; Kitamoto et al., 1987), and in Tetrahymena (Banno et al, 1987).

Since both high total and specific activities are essential in attempts to purify these enzymes, I have used five different cultural conditions to test their ability to influence the production of β-glucanase and β-glucosidase.

Materials and Methods

Culture of Organism

See Chapter 3 for basic cultural techniques and the five different cultural conditions.

Extraction of Enzymes

See Chapter 3 for the extraction of enzymes.

Assay of Enzyme Activity

The standard enzyme assay consisted of 0.4 ml substrate plus 0.1 mL enzyme solution, with incubation at 37 C for a suitable time, usually 20 to 60 min. For β-glucosidase activity, 2 mM p-nitrophenyl glucose (pNPG) in 50 mM Tris-HCl buffer, pH 7.6 was used as the substrate and the reaction was stopped by adding 2 mL 0.2 M Na_2CO_3. The product (p-nitrophenol) was measured at 410 nm and related to a standard curve of p-nitrophenol. One unit of β-glucosidase activity is defined as the liberation of 1 μmol p-nitrophenol min^{-1}. The units are given on the basis of 1 mL of enzyme solution. For β-glucanase activity, 1 mg mL^{-1} laminaran in 50 mM Tris-HCl buffer, pH 7.6 was used as the substrate, the resulting reducing sugar was measured by Somogyi's (1952) method with glucose as the standard. One unit of β-glucanase activity is defined as the liberation of 1 μmol reducing sugar min^{-1}. The units are given on the basis of 1 mL of enzyme solution.

Protein content in the enzyme solution and glucose in the culture medium were measured as described in Chapter 3.

Results

Mycelial Total and Estimated Specific Activities of β-glucosidase and β-glucanase from MEM.

In MEM medium, both β-glucanase and β-glucosidase showed an increased from 36 h to 84 h in total activity, followed by a decrease (Fig. 11). Similar increases in specific activity were seen up to 84 h followed by a small decrease (Fig. 12). The 84 h peak of total and specific activities of β-glucanase represents 6.3- and 8.9-fold increases (Figs. 11 - 12). The similar total and specific activities of β-glucosidase represents 2.8- and 4.1-fold increases (Figs. 11 - 12). Mycelial fresh weight and protein in the extracted enzyme decreased after 36 h (data not shown).

Mycelial Total and Estimated Specific Activities of β-glucosidase and β-glucanase from MEM-minus-Pi.

When the mycelium was transferred after 36 h on MEM to fresh MEM minus phosphate, the total activity of both β-glucanase and β-glucosidase increased up to 60 h before decreasing at 72 h, and this increase is 4- and 11.2-fold respectively (Fig. 13). The specific activity of both β-glucosidase and β-glucanase increased up to 72 h, and

representing increases of 2.8- and 7.4-fold (Fig. 14). The mycelial fresh weight doubled in 24 h after this transfer to fresh MEM-minus-phosphate, and then slightly decreased (data not shown). Glucose in the fresh cultural medium decreased by 85% in 12 h and was totally removed after 24 h (data not shown).

Mycelial Total and Estimated Specific Activities of β-glucosidase and β-glucanase Using Different Phosphate Concentrations.

The total activity of both β-glucosidase and β-glucanase increased with an increase in the phosphate concentration of the medium (Fig. 15). The specific activity of both enzymes were similar at all phosphate concentrations (Fig. 16). The fresh weight of the mycelium increased with an increase in phosphate concentration (data not shown).

Mycelial Total and Estimated Specific Activities of β-glucosidase and β-glucanase from the Lowest Phosphate Culture.

The total activity of β-glucosidase increased up to 60 h, then decreased (Fig. 17), whereas the specific activity increased up to 72 h before declining (Fig. 18). The total and specific activities of β-glucanase increased up to at 72 h before declining (Figs. 17- 18).

Mycelial Total and Estimated Specific Activities of β-glucosidase and β-glucanase During Ca^{+2}-induced Sporulation.

Total and specific activities of β-glucosidase increased sharply up to 8 h before decreasing, this represents a 7.2- and 6.5-fold increase respectively (Figs. 19 - 20). The total and specific activities of β-glucanase also increase sharply and peak at 8 h, this represents a 5.6- and 3.7-fold increase respectively (Figs. 19 - 20).

The highest total and specific activities of β-glucosidase and β-glucanase from each of the different cultural conditions are compared in Table 3.

Fig. 11. Total mycelial activities of β-glucosidase and β-glucanase of A. bisexualis on modified enriched medium (MEM). Growth conditions and enzyme extraction the same as in Fig. 1. p-Nitrophenyl glucose was the substrate for β-glucosidase activity, laminaran for β-glucanase activity. The assay consisted of 0.4 mL 2 mM substrate in 50 mM Tris-HCl buffer, pH 7.6 and 0.1 mL enzyme solution at 37 C. p-Nitrophenol was measured at 410 nm and related to a standard p-nitrophenol curve. One unit of β-glucosidase activity is defined as the liberation of 1 μmol p-nitrophenol min^{-1}. The units are given on the basis of 1 ml of enzyme solution . Reducing sugar was measured at 520 nm by the Somoygi method using glucose as the standard. One unit of β-glucanase activity is defined as the liberation of 1 μmol reducing sugar min^{-1}. The units are given on the basis of 1 ml of enzyme solution.

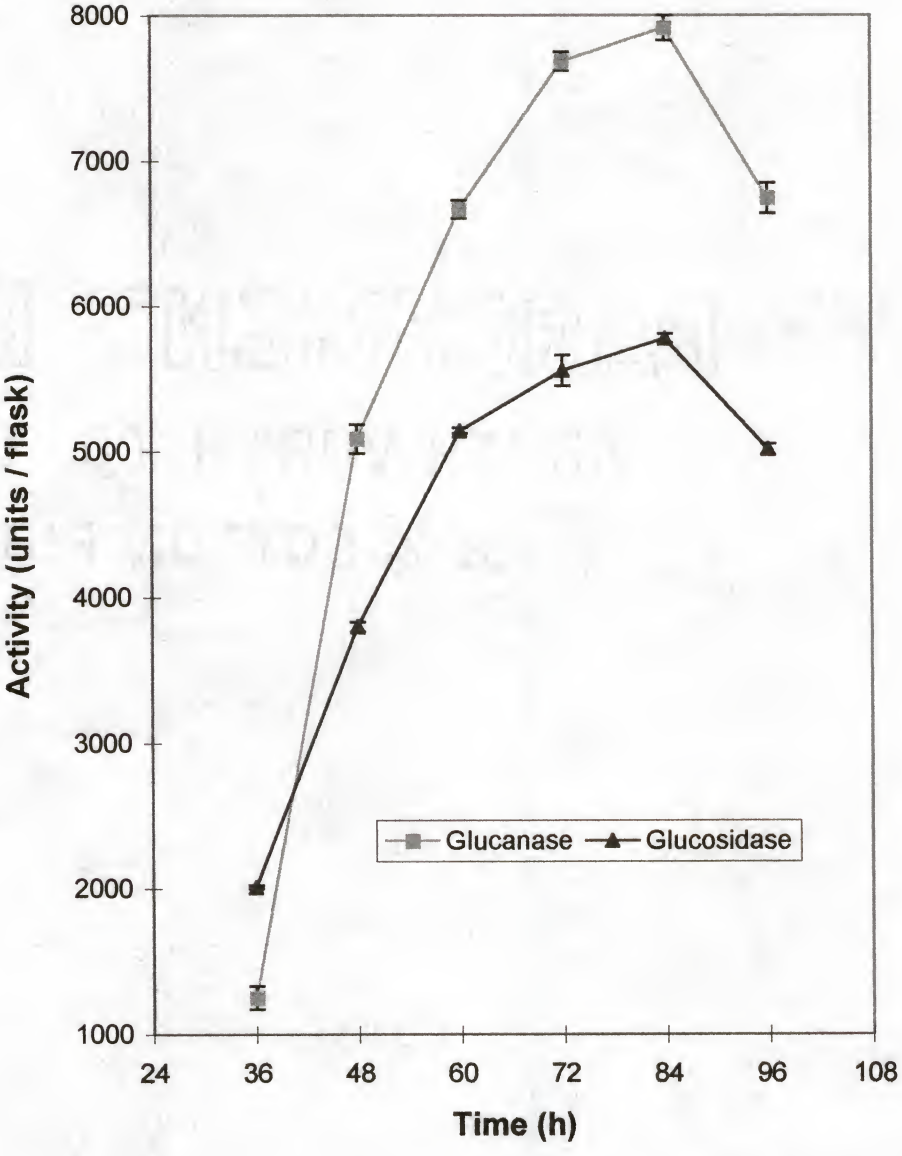

8000
7000
6000
5000
4000
3000
2000
1000
Activity (units / flask)
24
36
48
60
72
84
96
108
Time (h)
Glucanase
Glucosidase

Fig. 12. Estimated specific activities of β-glucosidase and β-glucanase of A. bisexualis on modified enriched medium (MEM). Experimental methods were the same as Fig. 11. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

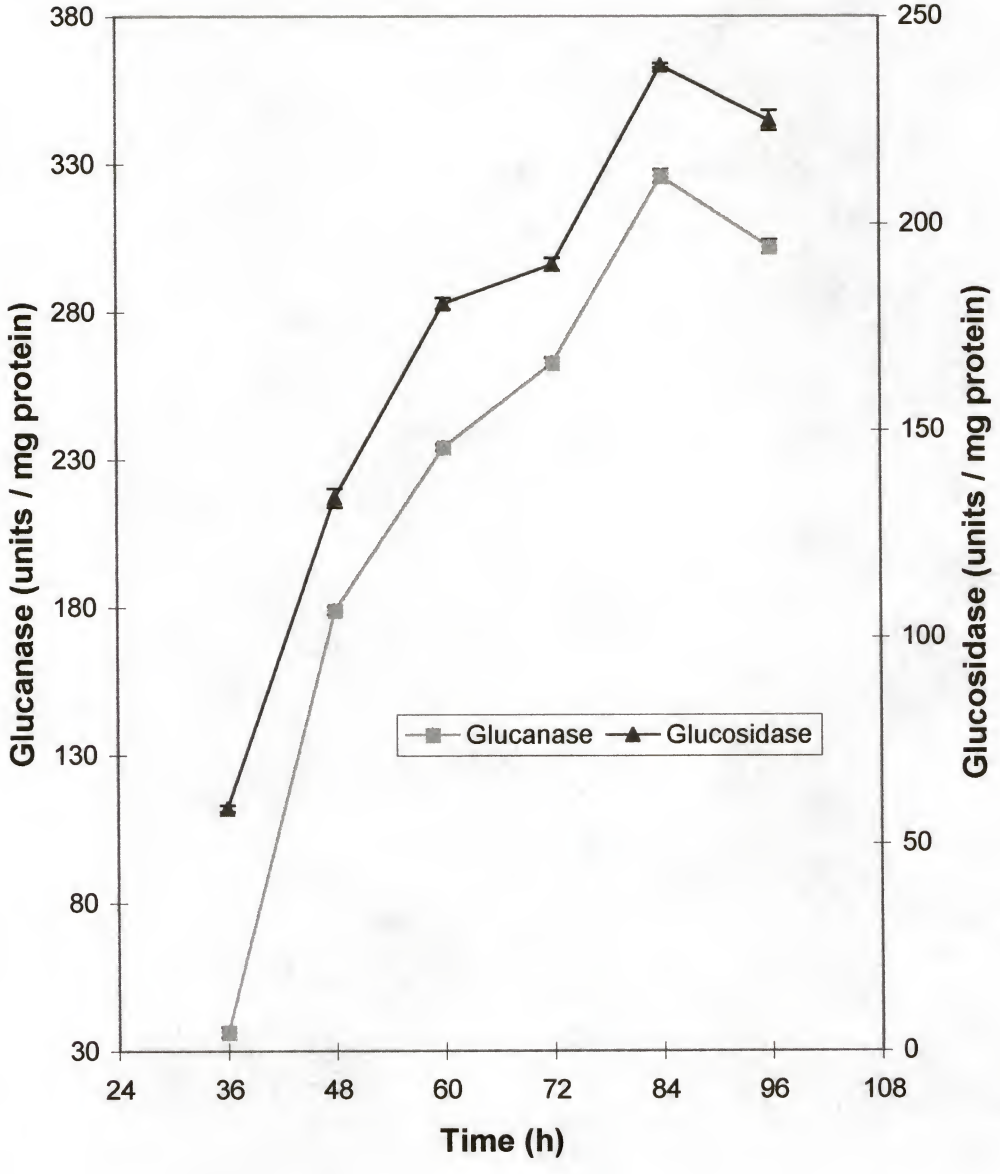

380
330
280
230
180
130
80
30
250
200
150
100
50
0
24
36
48
60
72
84
96
108
Glucanase (units / mg protein)
Glucosidase (units / mg protein)
Time (h)
Glucanase
Glucosidase

Fig. 13. Total mycelial activities of β-glucosidase and β-glucanase of A. bisexualis, Growth conditions were the same as Fig. 3. Enzyme extraction, assays and units were the same as Fig. 11.

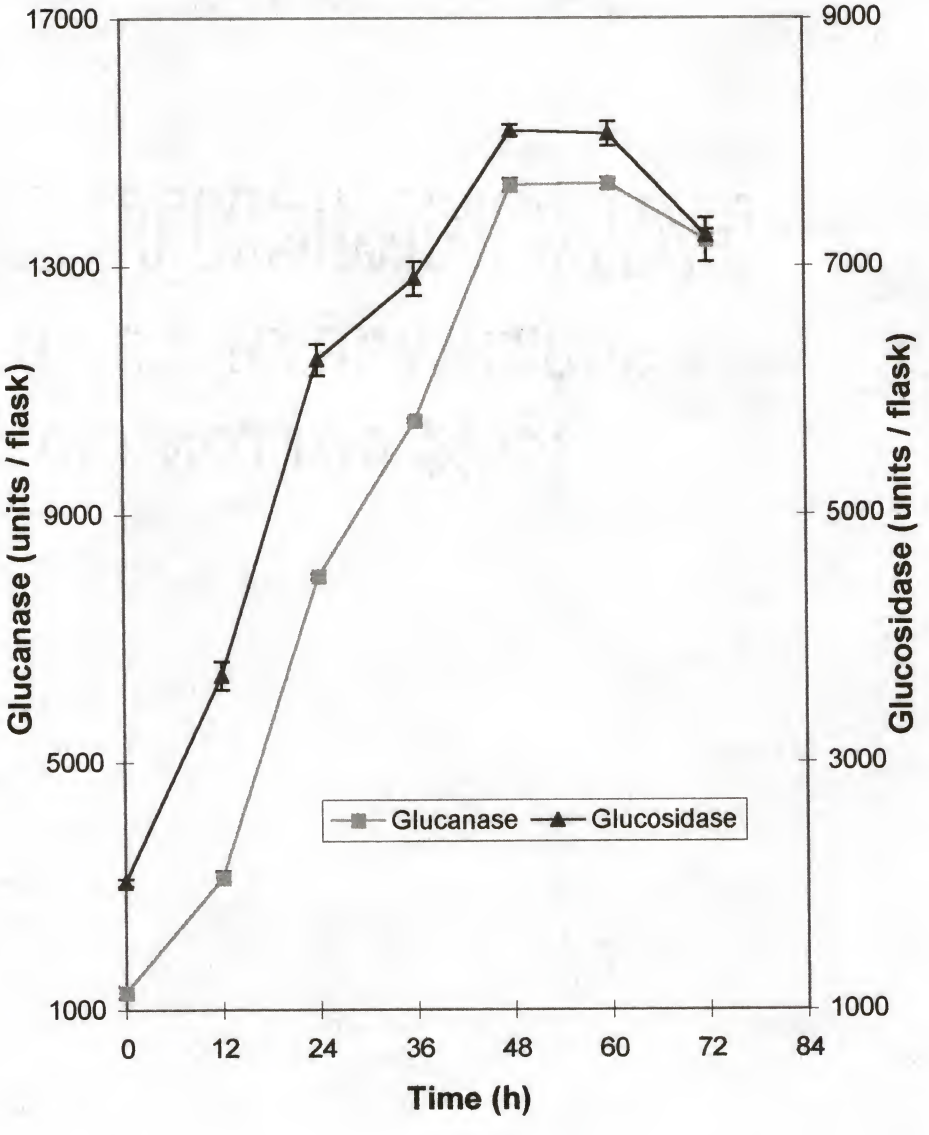

17000
13000
9000
5000
1000
9000
7000
5000
3000
1000
0
12
24
36
48
60
72
84
Glucanase (units / flask)
Glucosidase (units / flask)
Time (h)
Glucanase
Glucosidase

Fig. 14. Estimated specific activities of β-glucosidase and β-glucanase of A. bisexualis. Experimental methods were the same as Fig. 13. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

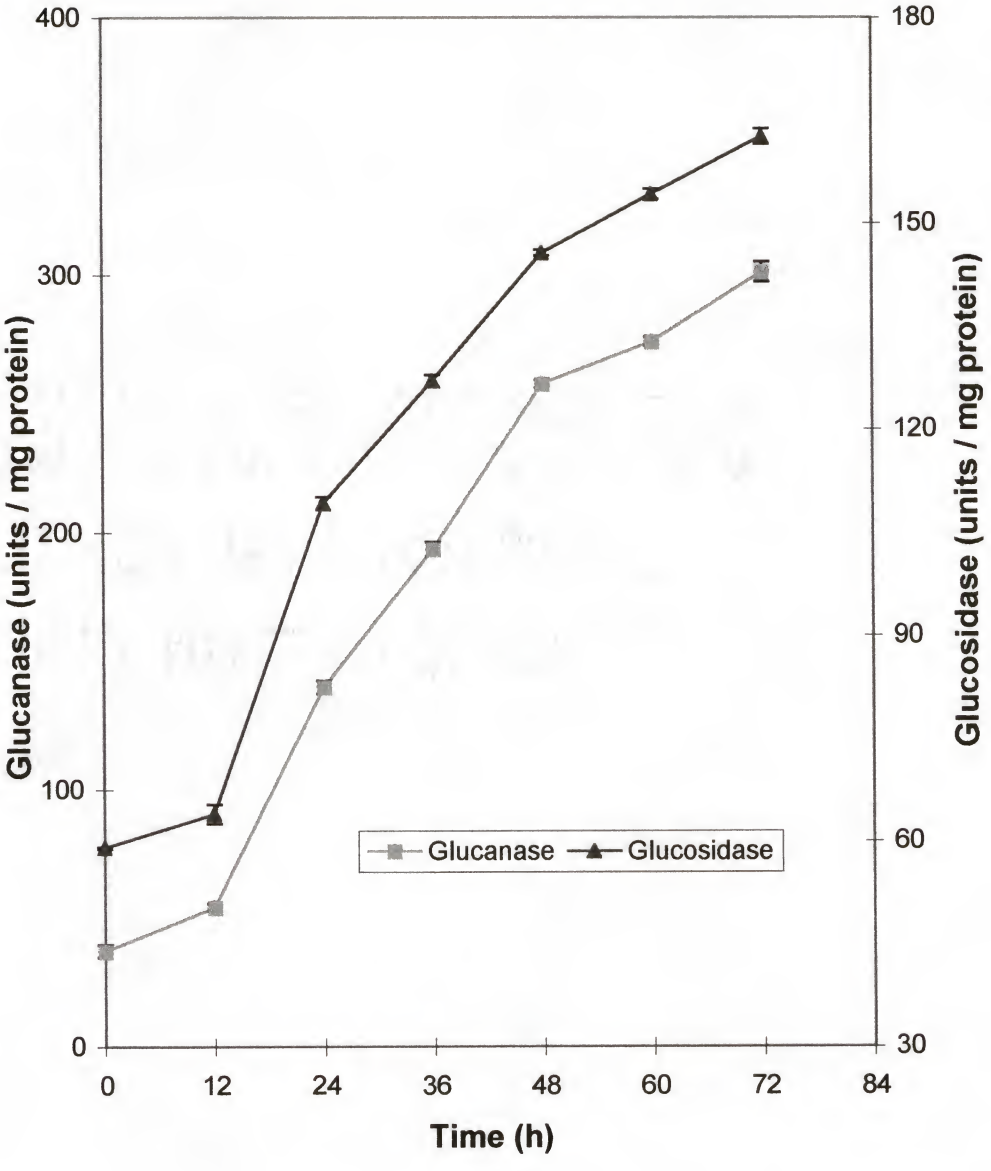

400
300
200
100
0
180
150
120
90
60
30
0
12
24
36
48
60
72
84
Glucanase (units / mg protein)
Glucosidase (units / mg protein)
Time (h)
Glucanase
Glucosidase

Fig. 15. Total mycelial activities of β-glucosidase and β-glucanase of A. bisexualis, Growth conditions were the same as Fig. 5. Enzyme extraction, assays and units were the same as Fig. 11.

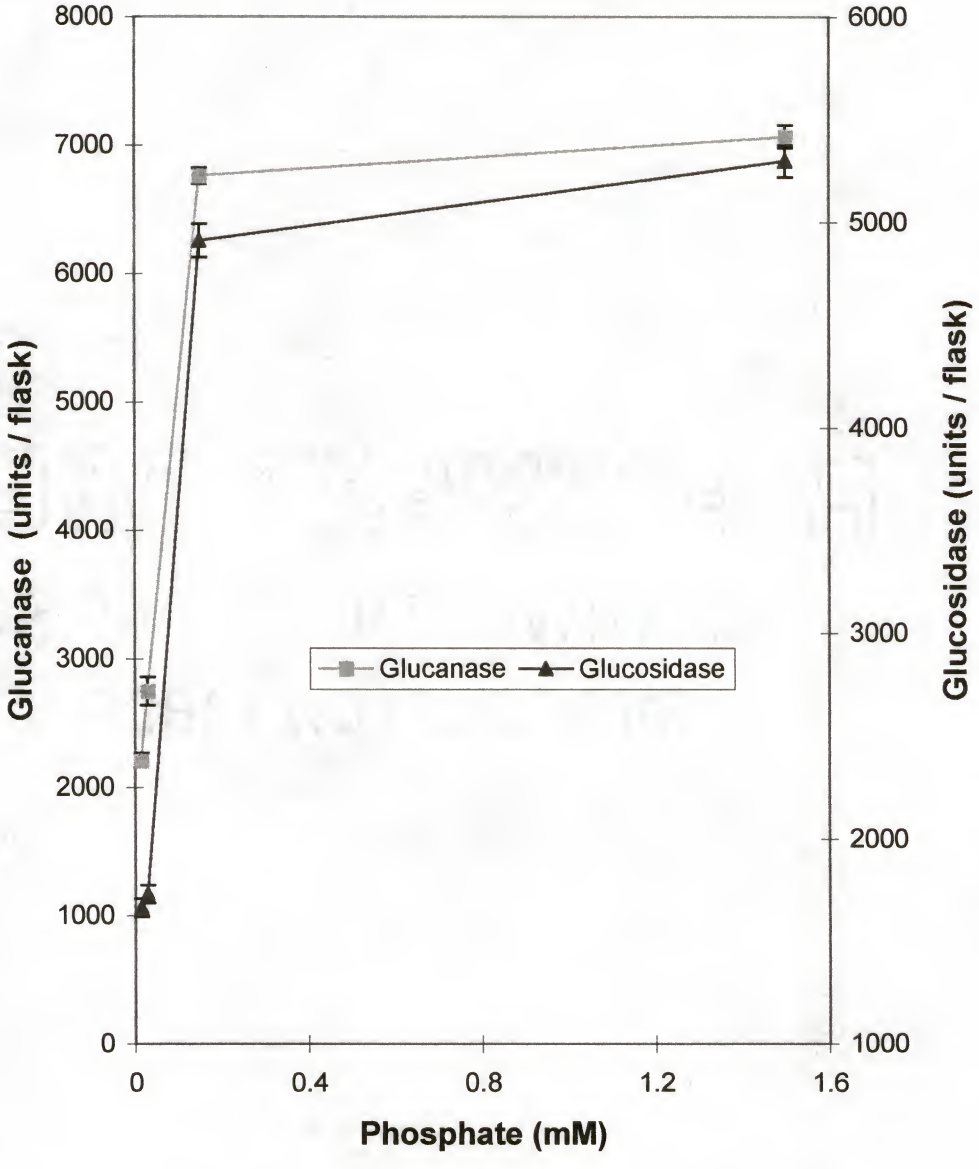

8000
7000
6000
5000
4000
3000
2000
1000
0
6000
5000
4000
3000
2000
1000
0
0.4
0.8
1.2
1.6
Glucanase (units / flask)
Glucosidase (units / flask)
Phosphate (mM)
Glucanase
Glucosidase

Fig. 16. Estimated specific activities of β-glucosidase and β-glucanase of A. bisexualis. Experimental methods were the same as Fig.15. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

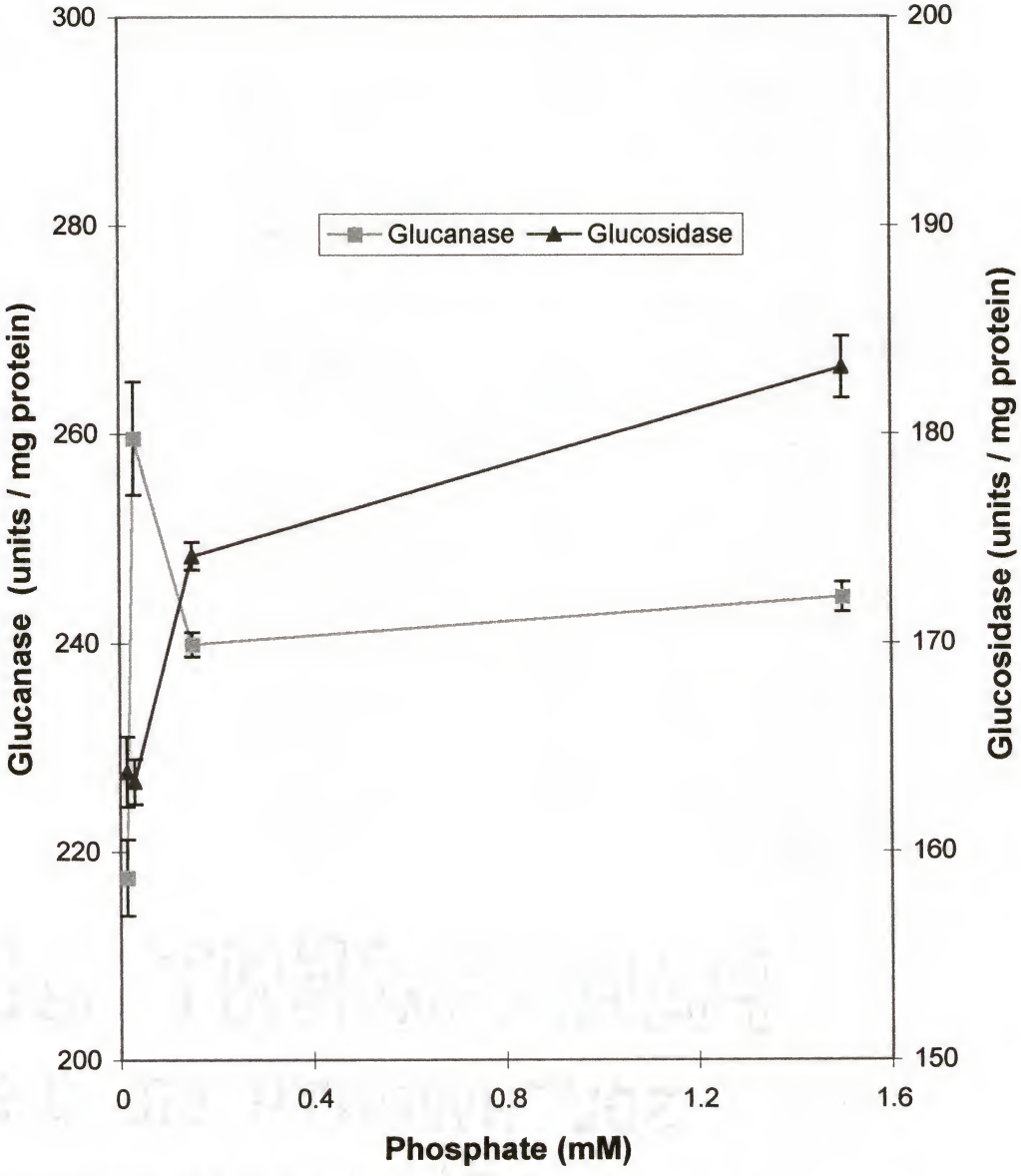

300
280
260
240
220
200
200
190
180
170
160
150
Glucanase
Glucosidase
Glucanase (units / mg protein)
Glucosidase (units / mg protein)
0
0.4
0.8
1.2
1.6
Phosphate (mM)

Fig. 17. Total mycelial activities of β-glucosidase and β-glucanase of A. bisexualis. Growth condition were the same as Fig. 7. Enzyme extraction, assays and units were the same as Fig. 11.

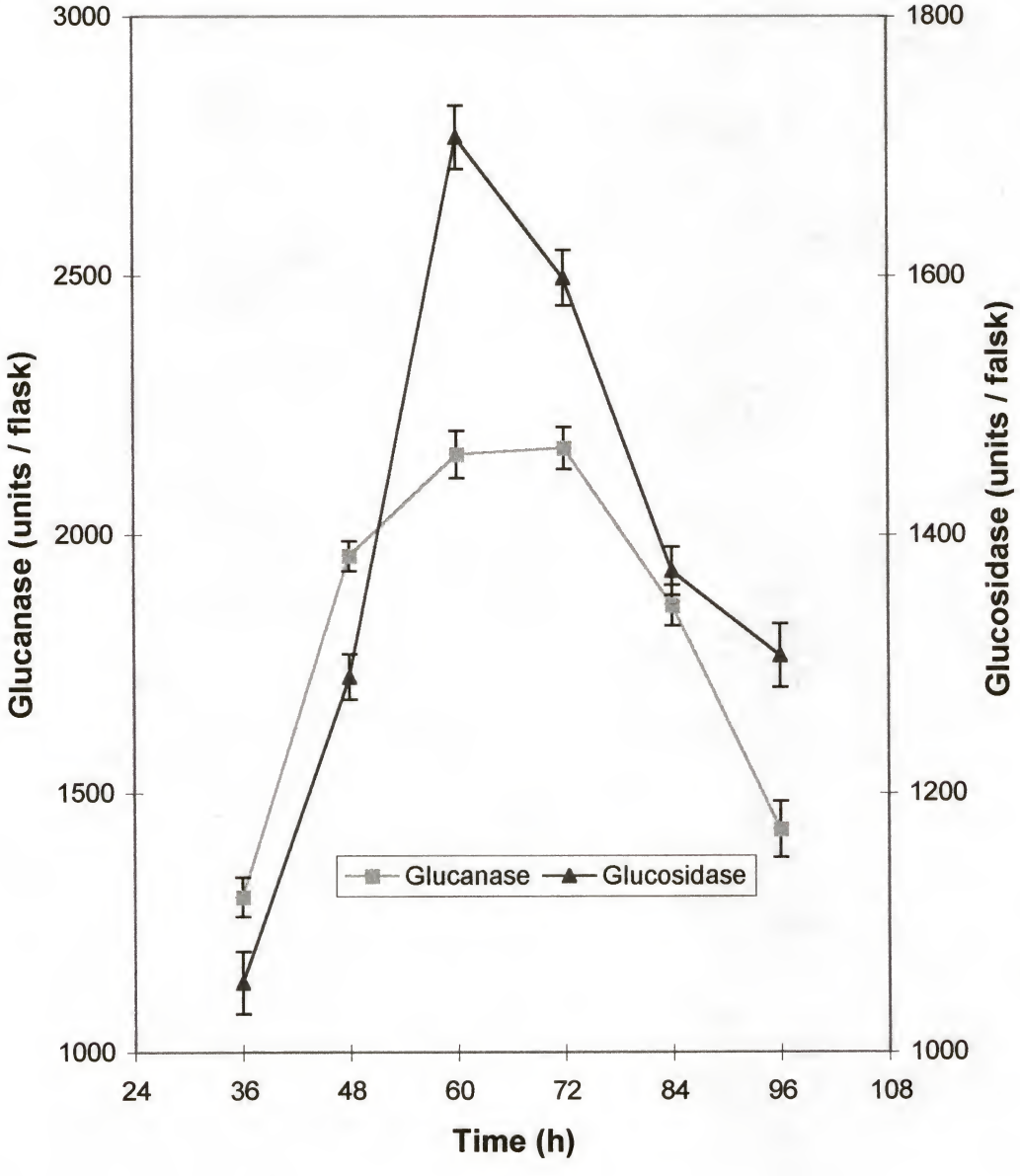

3000
2500
2000
1500
1000
1800
1600
1400
1200
1000
24
36
48
60
72
84
96
108
Glucanase (units / flask)
Glucosidase (units / falsk)
Time (h)
Glucanase
Glucosidase

Fig. 18. Estimated specific activities of β-glucosidase and β-glucanase of A. bisexualis. Experimental methods were the same as Fig. 17. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

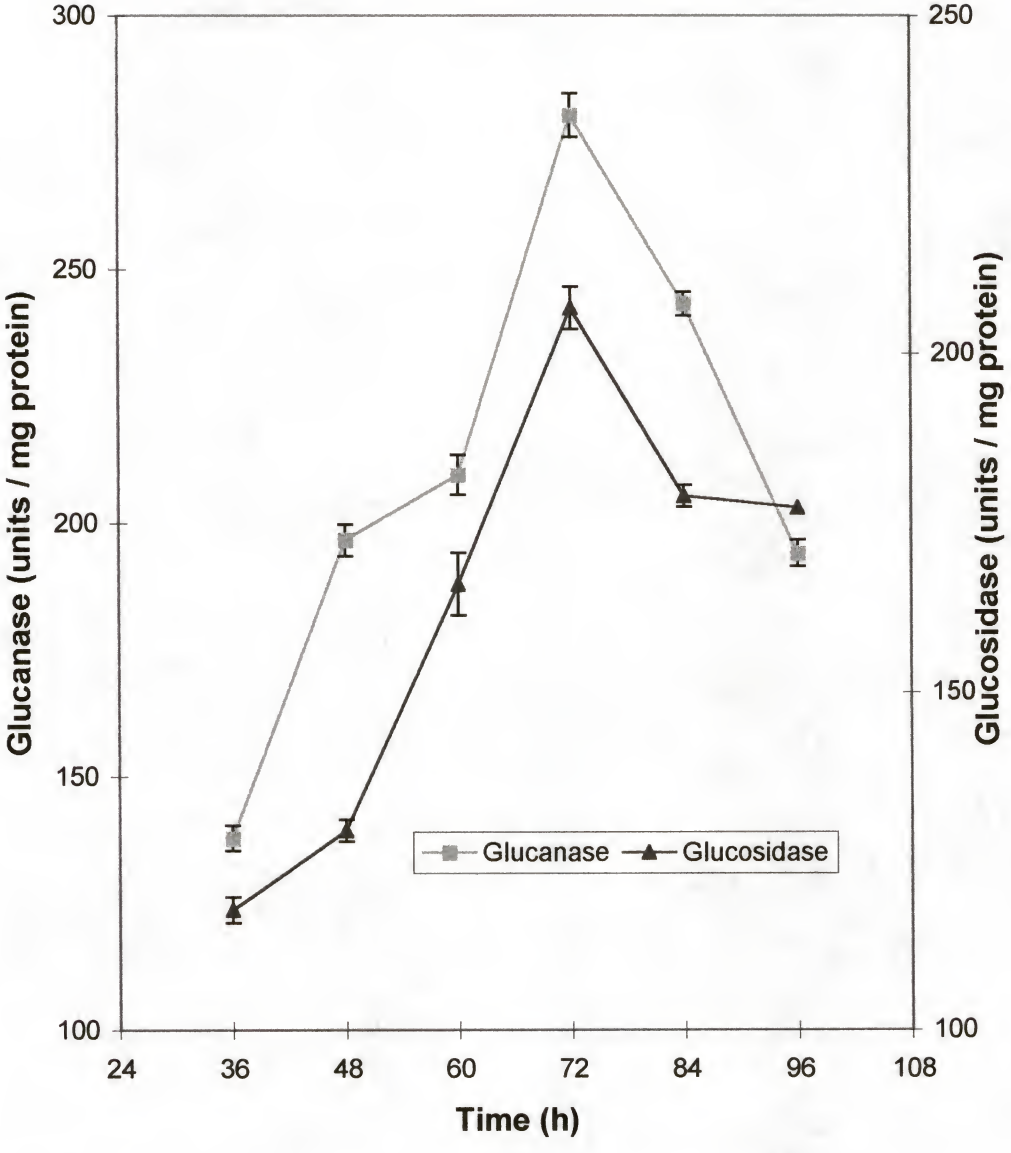

300
250
200
150
100
250
200
150
100
24
36
48
60
72
84
96
108
Glucanase (units / mg protein)
Glucosidase (units / mg protein)
Time (h)
Glucanase
Glucosidase

Fig. 19. Total mycelial activities of β-glucosidase and β-glucanase of A. bisexualis during Ca^{2+}-induced sporulation. Growth conditions were the same as Fig. 9. Enzyme extraction, assays and units were the same as Fig. 11.

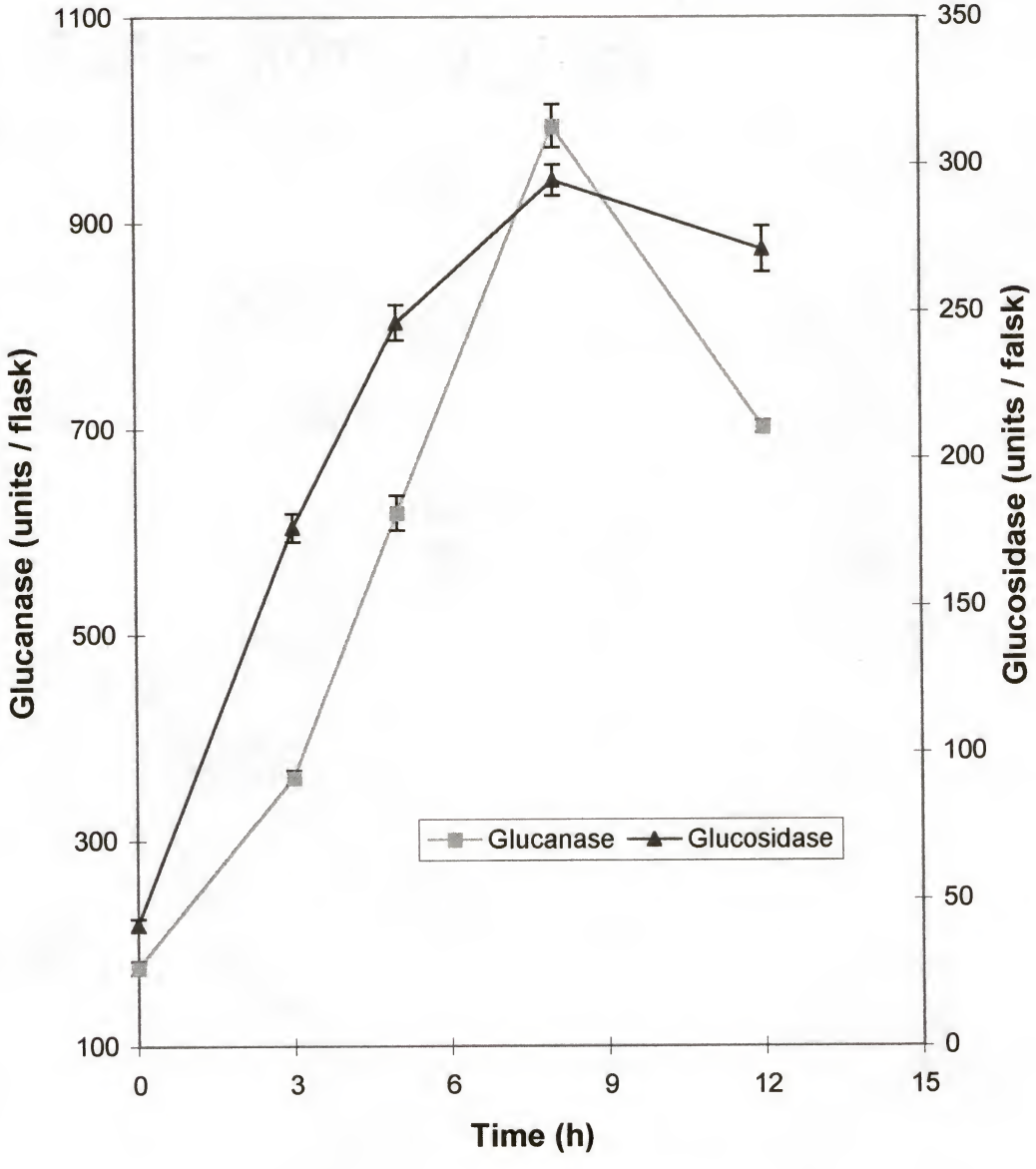

1100
900
700
500
300
100
350
300
250
200
150
100
50
0
0
3
6
9
12
15
Glucanase (units / flask)
Glucosidase (units / falsk)
Time (h)
Glucanase
Glucosidase

Fig. 20. Estimated specific activities of β-glucosidase and β-glucanase of A. bisexualis. Experimental methods were the same as Fig. 19. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

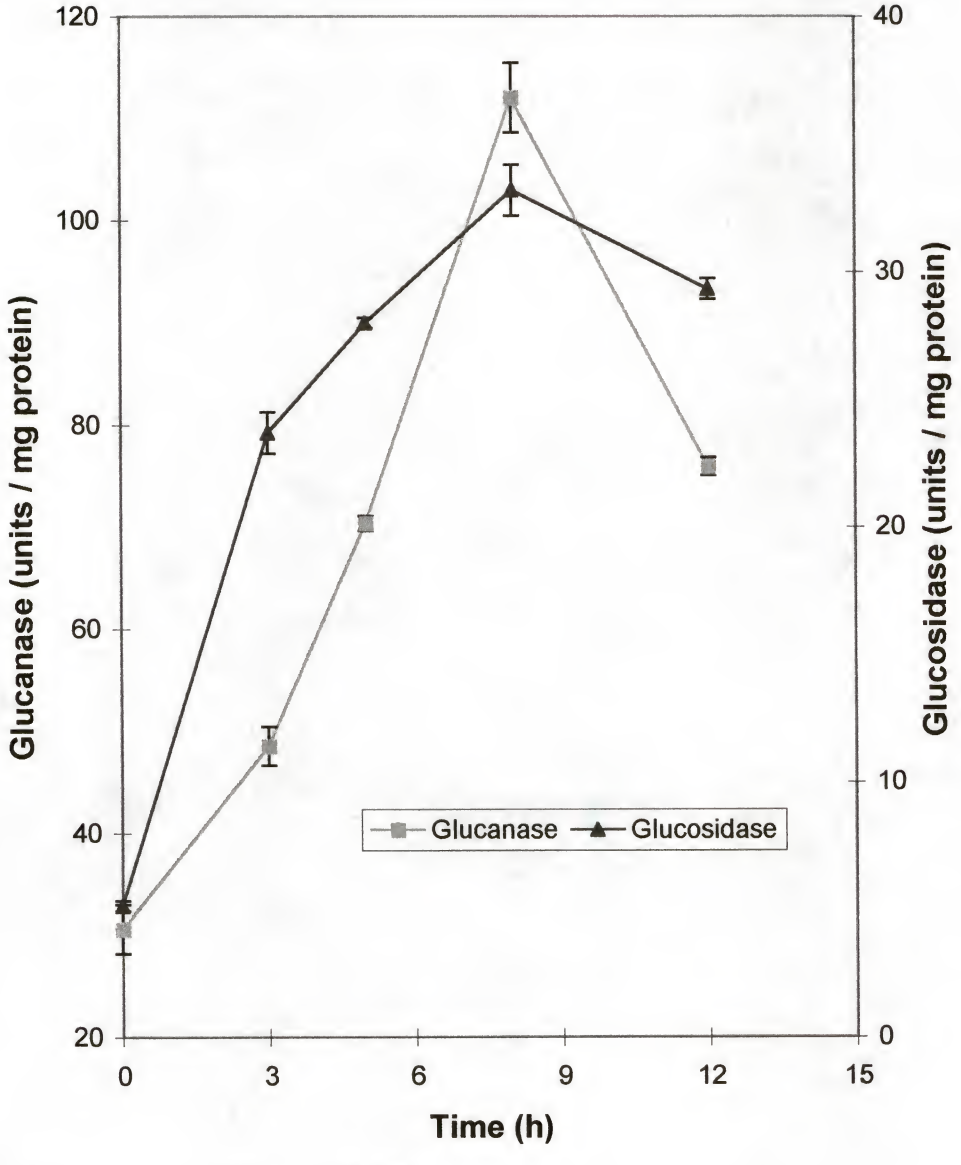

120
100
80
60
40
20
40
30
20
10
0
0
3
6
9
12
15
Glucanase (units / mg protein)
Glucosidase (units / mg protein)
Time (h)
Glucanase
Glucosidase

Table 3. The comparison of the highest total and specific activities of β-glucosidase and β-glucanase of the different cultural conditions

Medium[a]	Total Activity (Units flask^{-1}) β-glucosidase	Ratio[b]	β-Glucanase	Ratio[b]	Specific Activity (Units mg^{-1} protein) β-glucosidase	Ratio[b]	β-Glucanase	Ratio[b]
MEM	5789	--	7913	--	238	--	326	--
MEM-P	8093	1.40	14333	1.81	163	0.68	301	0.92
Different P	5298	0.92	7200	0.91	183	0.77	260	0.80
Low P	1706	0.29	2166	0.27	206	0.87	280	0.86
Sporulation	294.5	0.05	994.4	0.13	33.2	0.14	112	0.34

[a] Media are: MEM = modified enriched medium; MEM-P = MEM minus phosphate; Different P = MEM with phosphate concentrations of 0.015 - .5 mM; Low P = MEM with phosphate at 0.015 mM; Sporulation = 0.5 mM $CaCl_2$ solution.

[b] The ratio as compared with MEM.

Discussion

In MEM medium, it was previously shown that no glucose remains after 36 h, and continued growth utilizes the cytoplasmic reserve glucans, (Lee and Mullins, 1994). The results here demonstrate that the activity of β-glucanase is negligible up to 36 h. After 48 h, significant activities of both β-glucosidase and β-glucanase are present, and the production of these enzymes continues (Fig. 11). These results suggest that when the mycelium is carbon-starved, both β-glucanases and β-glucosidase activities are increased, and could be used for the mobilization of the cytoplasmic β-glucans.

Under MEM-minus-P medium, the mycelium was first cultured on MEM for 36 h, before being transferred to the medium lacking phosphate. It has been shown that when mycelium is cultured for 36 h in MEM, then supplemental glucose is added to the medium and the growth is continued for 24 h, the mycelial dry weight increases 58%, and mycelial glucan increases 135% (Lee and Mullins, 1994). These results demonstrate that when a supplemental carbon source is supplied, the mycelium responses with increased growth and accumulation of reserve materials. Using this same condition, the current results show that both mycelial growth and utilization of glucose are very fast, since after 12 h, the mycelial fresh weight almost doubles, and 85% of the supplemental glucose is removed (data not shown). At this point, β-glucosidase and β-glucanase activities are low. But at 24 h, when none of the

supplemental glucose remains, significant amounts of both enzyme activities are present.

Generally, the synthesis of these hydrolytic enzymes is markedly repressed when microorganisms are growing in a rapidly utilizable carbon source (Lilley and Ball, 1974; Friebe and Halldorf, 1975; Rey et al, 1979; Stantos et al., 1977; 1978; Rosa et al., 1984; Kitamoto et al., 1987; Rapp, 1989). This type of catabolite sugar repression is a widespread phenomenon in microorganisms (Gancedo, 1992; Saier, 1989). The result presented here adds another example to such a phenomenon.

When mycelium is cultured in MEM with different phosphate concentrations, the total activities of both β-glucosidase and β-glucanase increase with an increase in phosphate (Figs. 15-16). Mycelial growth also increases with an increase in phosphate supply (data not shown).

A growth kinetic experiment was designed for the lowest phosphate (0.015 mM) medium. In this medium, growth is slower as compared with MEM, glucose is not totally removed until 72 h and mycelial fresh weight is about 50 % of the expected (data not shown). Significant total β-glucanase activity, however, is present at 60 h, representing a 1.7-fold increase when compared with 36 h under MEM, and the estimated specific activity is 5.8-fold higher. These results indicate that the production of this activity is not induced by carbon starvation, but by phosphate starvation. This may be the first example of a β-glucanase which reaches to its highest level under phosphate starvation. Considering the structure of the cytoplasmic glucan, it may suggest that under phosphate starvation, β-glucanases would be activated, along with

phosphomonoesterases and phosphodiesterases, to cooperatively mobilize both carbon and phosphate.

Another interesting observation under this low phosphate condition is that the production of both types of enzymes appear not be responsive to carbon starvation, once the mycelium is limited by phosphate. No significant amount of β-glucanase activity is produced after 72 h, and the production of β-glucosidase decreases (Figs. 17-18).

Interactive effects between carbon and phosphate have been reported. In A. bisexualis, the accumulation of total reserve β-glucans decreases by 50% under low phosphate concentration (Lee and Mullins, 1994). In suspension-cultured Catharanthus cells, the rate of carbon uptake and incorporation of smaller sugars into larger ethanol-soluble fractions are lower in phosphate-deficient medium. This result suggests that phosphate strongly affects the utilization of sugars by cultured plants cells through both the stimulation of sugar transport and the activation of metabolism (Li and Ashihara, 1989). The catabolism of glucose via both glycolysis and the pentose phosphate pathway is stimulated and enhanced by phosphate, mainly due to the increase in levels of ATP, $NADP^+$ and other phosphorylated compounds (Li and Ashihara, 1990). In Brassica suspension cells, a phosphate starvation responsive (prs3) gene which encodes a β-glucosidase is expressed under phosphate starvation, and the suggested role of this β-glucosidase is in the deglycosylation and regulation of phosphatases and other enzymes (Malboobi and Lefebvre, 1995).

Under Ca^{2+}-induced sporulation, the mycelium may be forced to utilize its reserve materials to support spore production. The increases in both β-glucosidase and β-glucanase

activities suggest that reserve materials may be mobilized to support this new morphogenesis. High β-glucanase activity during sporulation is reported in many species (Fleet and Phaff, 1975; Villa et al., 1978; Del Rey et al, 1979; Rey et al., 1979; Reichlet et al., 1981; Rey et al., 1982; Hien and Fllet, 1983; Cottingham and Mullins, 1985; Muthukumar et al., 1993). Sporulation-specific β-glucanases are reported in Schizosaccharomyces (Reichlet et al., 1981), and in Saccharomyces (Rey et al., 1979). These enzymes are absent in vegetative cells and synthesized only during sporulation. Recently, a gene which encodes a sporulation-specific β-glucanase is described in Saccharomyces (Muthukumar et al., 1993).

In summary, the highest activities of both β-glucosidase and β-glucanase are produced after the downshift from MEM to MEM-minus-P, mainly because of the larger mycelial mass. The highest specific activities of both β-glucosidase and β-glucanase were with MEM (Table 3). These results suggest that carbon starvation exerts a major influence over the production of β-glucosidase and β-glucanase in A. bisexualis. A striking observation was found under phosphate limitation (0.015 mM), where significant levels of β-glucanase and β-glucosidase are produced in the presence of glucose, and both activities are no longer responsive to carbon starvation. These results suggest that under low phosphate, phosphate rather than carbon is the major nutrient that influences β-glucosidase and β-glucanase activities in A. bisexualis. This appears to be the first example of a β-glucanase which reaches to its highest level under phosphate starvation. This finding opens up the possibility for studying the regulatory mechanism for this phenomenon.

Originally, the sporulation condition was thought to be the most effective way to promote the production of β-glucosidase and β-glucanase, but these results show that the total mycelial activities of β-glucosidase and β-glucanase are very low, mainly as a result of the small mycelial mass used for sporulation as mentioned in previous chapter.

Chapter 5
PURIFICATION AND CHARACTERIZATION OF A β-(1->3)-GLUCANASE FROM ACHLYA DURING CA^{2+}-INDUCED SPORULATION

Introduction

The results in Section II revealed two nutrient conditions which influence the production of β-(1->3)-glucanases in Achlya. In one system, the production of β-(1->3)-glucanases occurs under by carbon limitation, such as the complete removal of glucose from the cultural medium. Four different β-(1->3)-glucanases, two extracellular activities which are secreted into the medium, and two intracellular activities were found. In the other cultural system, the β-(1->3)-glucanase activities are suggested to be regulated by phosphate limitation, where β-(1->3)-glucanase activities are detected in the presence of glucose, but under phosphate starvation.

A β-(1->3)-glucanase has been purified from Achlya mycelium under the condition of Ca^{2+}-induced sporulation and its action has been characterized against a number of substrates, including extracted cytoplasmic β-(1->3)-glucans from Achlya which appear to be its cellular substrates.

Materials and Methods

Culture of Organism

See Chapter 3 for basic cultural techniques and Ca^{2+}-induced sporulation.

Extraction of Enzymes

See Chapter 3 for the initial extraction of enzymes.

Enzyme Purification

The extraction and all subsequent procedures were performed at 4 C. The crude enzyme supernatant was first fractionated by 70% $(NH_4)_2SO_4$. Precipitated protein was redissolved in 25% $(NH_4)_2SO_4$ in 50 mM sodium acetate buffer, pH 5.0 containing 0.1% Micro-o-Protect, an anti-bacteria agent from Boehringer Mannheim, (buffer A), and applied to a 2.5 x 30 cm column of phenyl-Sepharose CL-4B(Sigma) previously equilibrated with 25% $(NH_4)_2SO_4$ in buffer A. Unbound proteins were removed with a decreasing gradient of 25% - 0% $(NH_4)_2SO_4$ in buffer A. Bound proteins were eluted first with a linear gradient of 0 - 50% ethylene glycol in buffer A, followed by 6 M urea elution in the same buffer. Two β-(1->3)-glucanases activity peaks were obtained, one from the glycol elution and another from the urea elution. Each was pooled and dialyzed against buffer A overnight. The enzyme eluted by glycol was then applied to a 2.5 x 30 cm CM-Sepharose fast-flow column (Sigma) previously equilibrated in buffer A, and eluted with a linear gradient of 0 - 0.5 M NaCl in buffer A, active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 7.5 with 0.1% Micro-o-Protect (buffer B). This activity was then added to a 2.5 x 30 cm column

of Cibacron blue 3 GA (Sigma) previously equilibrated in buffer B, for the efficient removal of phosphatase activities. The unbound β-(1->3)-glucanase was applied to a 2.5 x 30 cm DEAE-cellulose column (Sigma) that had been equilibrated in buffer B, and eluted with a linear gradient of 0 - 0.5 M NaCl in buffer B. Peak fractions of β-(1->3)-glucanase activity were pooled and concentrated in a pressure cell (Amicon Corp.). The concentrated activity was finally applied to a 1 x 50 cm Sephadex G-200 column (Pharmacia) previously equilibrated in buffer B, activity peak fractions were collected and concentrated by ultrafiltration (Amicon Corp.). These purification steps are outlined in Fig. 21.

This β-(1->3)-glucanase activity was tested for purification by SDS-PAGE electrophoresis. Electrophoresis was performed using precasted gels on a Ready Gel Cell (Bio-RAD) using reagents and procedures obtained from Bio-RAD. The β-(1->3)-glucanase activity from the urea elution was also subjected to the above procedures, and tested for purity by SDS-PAGE.

Assay of Enzyme Activity

The standard enzyme assay consisted of 0.4 mL substrate plus 0.1 mL enzyme solution, with incubation at 37 C for a suitable time, usually 20 to 60 min. For β-glucanase activity, 1 mg mL^{-1} laminarin (from Laminaran digitata, Sigma) in 50 mM sodium acetate buffer, pH 6.0 was the substrate, and the resulting reducing sugar was measured by Somogyi's (1952) method with glucose as the standard. One unit of β-glucanase activity is defined as the liberation of 1 μmol reducing sugar min^{-1}. The units are given on the basis of 1 mL of enzyme solution.

Protein content in the enzyme solution was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Protein during column elutions was monitored by absorbance at 280 nm. Glucose in the culture medium or as an enzyme hydrolysis product was measured by the glucose oxidase-peroxidase method (Gascon et al., 1968), with glucose as the standard.

Effect of pH on Activity and Stability

The purified β-(1->3)-glucanase was tested for effect of pH on its activity and stability. Buffer solutions were prepared to cover the pH range of 4 - 11. They were 50 mM sodium acetate for pH 4 - 6; 50 mM phosphate for pH 6.5 - 7.5; 50 mM Tris-HCl for pH 8 - 9; and 50 mM carbonate/bicarbonate for pH 10 -11. Substrate was dissolved in each buffer and the activity was measured under the standard condition for 1 h. The effect of pH on enzyme stability were observed by assaying the enzyme activity after incubation at 25 C or 4 C for 24 h without substrate at each of the same assay.

Effect of Temperature on Activity and Stability

The purified β-(1->3)-glucanase activity was measured at various temperatures (20 - 70 C) under the standard condition for 1 h. The heat stability of the enzyme was determined by assaying the activity after incubation for 1 h at various temperatures in the absence of substrate. Long term storage stability was measured by assaying the enzyme activity under the standard assay condition after storing the enzyme at 4 C for 1, 2 and 7 days in the absence of substrate.

Effect of Metal Ions on Activity

The purified β-(1->3)-glucanase was incubated at 25 C for 30 min in the presence of the following metal ions at 0.1, 1 and 5 mM: $CaCl_2$, $CuSO_4$, $CoCl_2$, $FeCl_3$, $FeSO_4$, $HgCl_2$, $LaCl_3$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$. Activity was measured by the standard assay. A cocktail of 0.2 mM EDTA, DTT and PMFS used to inhibit proteases during purification was also used to determined their effect on the enzyme activity.

Substrate Specificity and Action Pattern

A range of oligo- and polysaccharides, differing in their linkage was used to determined substrate specificity. Each substrate was prepared in 50 mM sodium acetate buffer, pH 6.0 at 1 mg mL^{-1}. The assay was carried out at 37 C for 1 h, and the resulting reducing sugar or glucose was measured as previously described. Control assays measured substrates only, which was then subtracted from enzyme assays.

The range of reaction products of the purified β-(1->3)-glucanase on laminarin, neutral glucan and phosphoglucan from Achlya were examined by thin layer chromatography (TLC). Single dimension ascending TLC was performed on precoated silica plates (Fisher) in a solvent saturated tank at room temperature. To minimize the salt effect on TLC, the concentration of the buffer was reduced from 50 mM to 10 mM. The assay used 4 mL of 2 mg mL^{-1} substrate in 10 mM sodium acetate buffer, pH 6.0 and 1 mL enzyme at 37 C. Aliquots of 1 mL of the reaction mixture were removed at 20, 60 min, 12 and 24 h. The reaction was stopped by boiling at 100 C for 5 min. 10 μL of the sample was applied to the plate and dried with a stream of cool air.

The solvent system was n-butanol-acetic acid-ethyl ether-water (9:6:3:1) (v/v). Carbohydrates were detected by spraying with phenol-sulfuric acid (Touchstone, 1992).

Kinetic Properties of the β-(1->3)-glucanase

Kinetic properties (K_m, V_{max}) were determined under standard assay condition for 1 h. Substrates (Laminaran digitata laminarin, crude glucan, phosphoglucan and neutral glucan from Achlya) were prepared in 50 mM sodium acetate buffer, pH 6.0 in the range of 0.25 - 2.5 mg mL^{-1}. K_m and V_{max} values were calculated from a Lineweaver-Burk plot.

Results

Purification of the β-(1->3)-glucanase

The purification profile using the steps outlined in Fig. 21 is summarized in Table 4. Ammonium sulfate fractionation of the crude supernatant gave a recovery of 85 % of the initial activity. This activity was applied to a hydrophobic interaction column. Unbound proteins were washed from this column using a decreasing salt gradient, and two β-(1->3)-glucanase activities were separated, one with glycol and corresponding to fraction numbers 28 - 34, the other with urea corresponding to fraction numbers 48 - 54 (Fig. 22). The two activities were pooled separately for further purification.

The pooled and dialyzed activity from the glycol elution was applied to a cation exchange column, and eluted with a linear salt gradient. Activity was eluted early in fraction numbers 9 - 12 (Fig. 23). This activity was pooled and dialyzed against buffer B to change buffer (Scopes, 1987), and applied to a Cibacron blue 3 GA column which will bind phosphatases. The unbound β-(1->3)-glucanase activity was applied to an anion exchange column, and elution with a linear salt gradient, gave activity in fraction numbers 8 - 12 (Fig. 24). This activity was pooled and concentrated then subjected to gel filtration, where the activity peaked at fraction number 18 (Fig. 25). This peak fraction gave a single band on SDS-PAGE (Fig. 26).

The fractions containing β-(1->3)-glucanase activity from the urea elution of the hydrophobic interaction column were subjected to the same procedure, but purification was

not obtained. The main activity appears to be an endo type based on the reaction products (data not shown).

Effect of pH on Activity and Stability

The optimal pH of the β-(1->3)-glucanase was at pH 6 (Fig. 27). The activity profile is narrow, with maximum activity between pH 6 - 7. Activity is lost at pH 9 and above and below pH 4. The pH stability range is also narrow for storage at 4 C and 25 C (Fig. 27).

Effect of Temperature on Activity and Stability

The optimal temperature for the activity was at 40 C, and activity decreased sharply after 45 C (Fig. 28). Temperature stability and activity of the enzyme were similar. This enzyme is stable at 4 C for at least 7 days (data not shown).

Effect of Metal Ions on Activity

The effect of metal ions on the β-(1->3)-glucanase is summarized in Table 5. The metal ions, Cu^{2+}, Hg^{2+} and Fe^{3+} strongly inhibited activity at all concentrations used. Moderate inhibition occurred with Fe^{2+} and Zn^{2+} at low concentrations and increased at higher concentration. Slight inhibition occurred at low Mn^{2+}, Co^{2+} and La^{3+} concentrations and moderate at higher concentration. Activity increased with Mg^{2+} at low concentrations and slight inhibition at higher concentration. The enzyme tolerates Ca^{2+} at the high concentration of 5 mM. The protease inhibitory cocktail used during purification showed no inhibition at the concentration used, but moderate inhibition at higher concentrations.

Substrate Specificity and Action Pattern of the β-(1->3)-glucanase

The activities of the β-(1->3)-glucanase on a range of putative substrates are compared in Table 6. The only substrates giving activity were the cytoplasmic glucans of Achlya, laminarin and laminaribiose. The highest activity occurred with laminarin and neutral glucan. The phosphoglucan and laminaribiose were poor substrates. No activity with pachyman or schizophyllan.

The action pattern of the β-(1->3)-glucanase was determined by separating the products released from the hydrolysis of laminarin, neutral glucan and phosphoglucan on TLC. The initial products were mainly glucose, with a trace amount of laminaribiose, later glucose was the only product (Figs. 29 - 31). This action pattern is typical of an exohydrolase and warrants the classification of the β-(1->3)-glucanase as an exo-β-(1->3)-D-glucan glucohydrolase (EC 3. 2. 1. 58).

Kinetic Properties of the β-(1->3)-glucanase

The purified β-(1->3)-glucanase shows hyperbolic Michaelis- Menten kinetics over the concentration range of 0.25 - 2.5 mg mL^{-1} for the substrates used (Fig. 32). The K_m values (mg mL^{-1}) calculated from these data using a Lineweaver-Burk plot were 0.67 for laminarin, 1.11 for neutral glucan, 2 for crude glucan and 2.1 for phosphoglucan. Calculated V_{max} values (mmol min^{-1} mg^{-1} protein) were 13.3 for laminarin, 11.7 for neutral glucan, 10.1 for crude glucan and 10 for phosphoglucan.

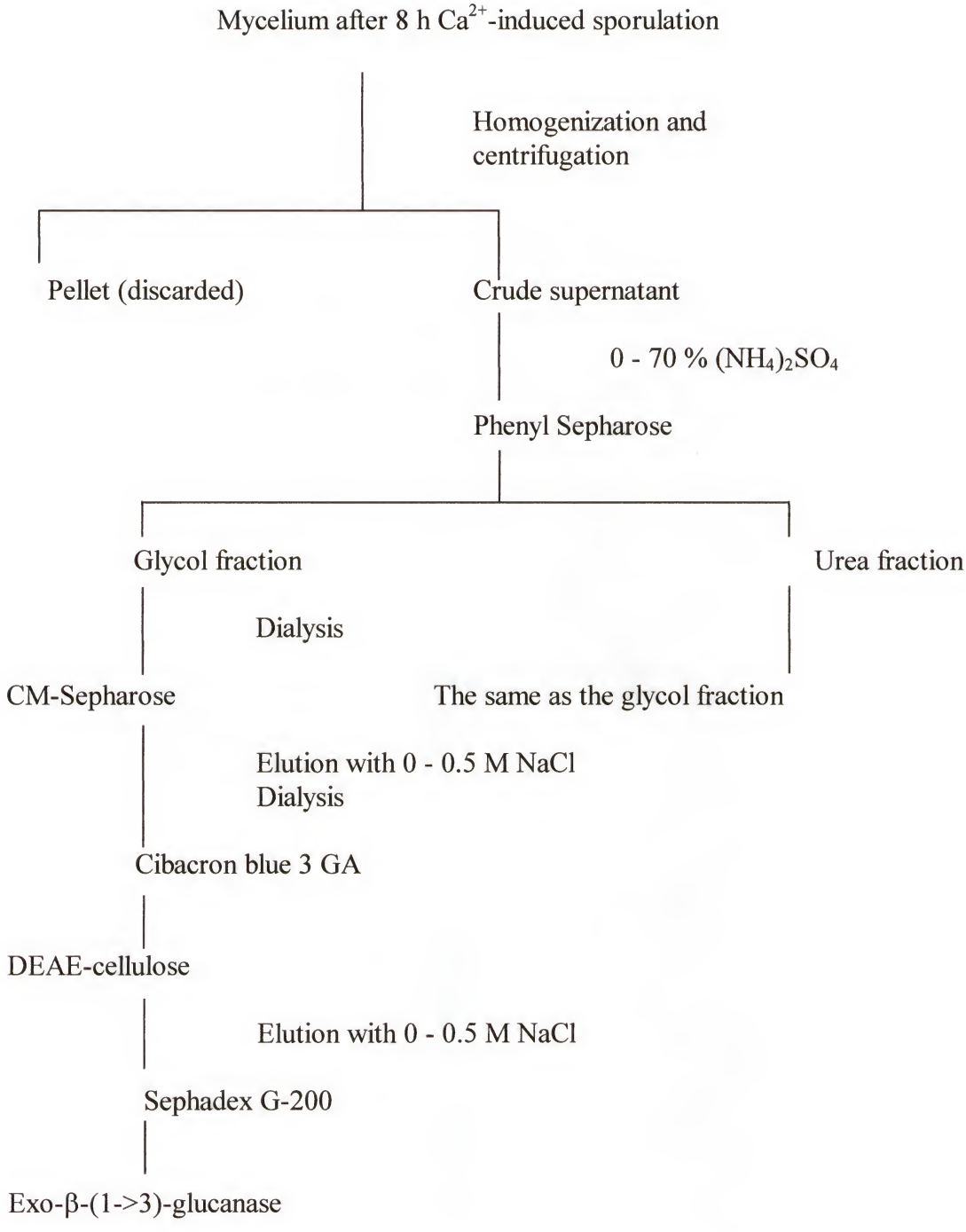


Fig. 21. Summary of purification procedures of the β-(1->3)-glucanase

Table 4. Purification of the β-(1->3)-glucanase

	Protein (mg)	Activity (units)	Specific Activity (units mg^{-1} protein)	Yield (%)	Purification Fold
Crude supernatant	350	25200	72	100	1
0 - 70% $(NH_4)_2SO_4$	185	21420	116	85	1.6
Phenyl-Sepharose	25.5	10332	405	41	5.6
CM-Sepharose	3.80	7182	1890	29	26.3
Cibacron blue 3 GA	3.00	6300	2100	25	29.2
DEAE-cellulose	1.10	3931	3574	16	49.0
Sephadex G-200	0.09	706	7840	2.8	108.9

Fig. 22. Separation of β-(1->3)-glucanases from 70% $(NH_4)_2SO_4$ fractionation on hydrophobic interaction column. Proteins were applied to the column, washed with 25 - 0% $(NH_4)_2SO_4$ in buffer A, and eluted with a linear gradient of 0 - 50% glycol followed by 6 M urea in buffer A. Absorbance at 280 nm is protein, and at 520 nm is β-(1->3)-glucanase activity.

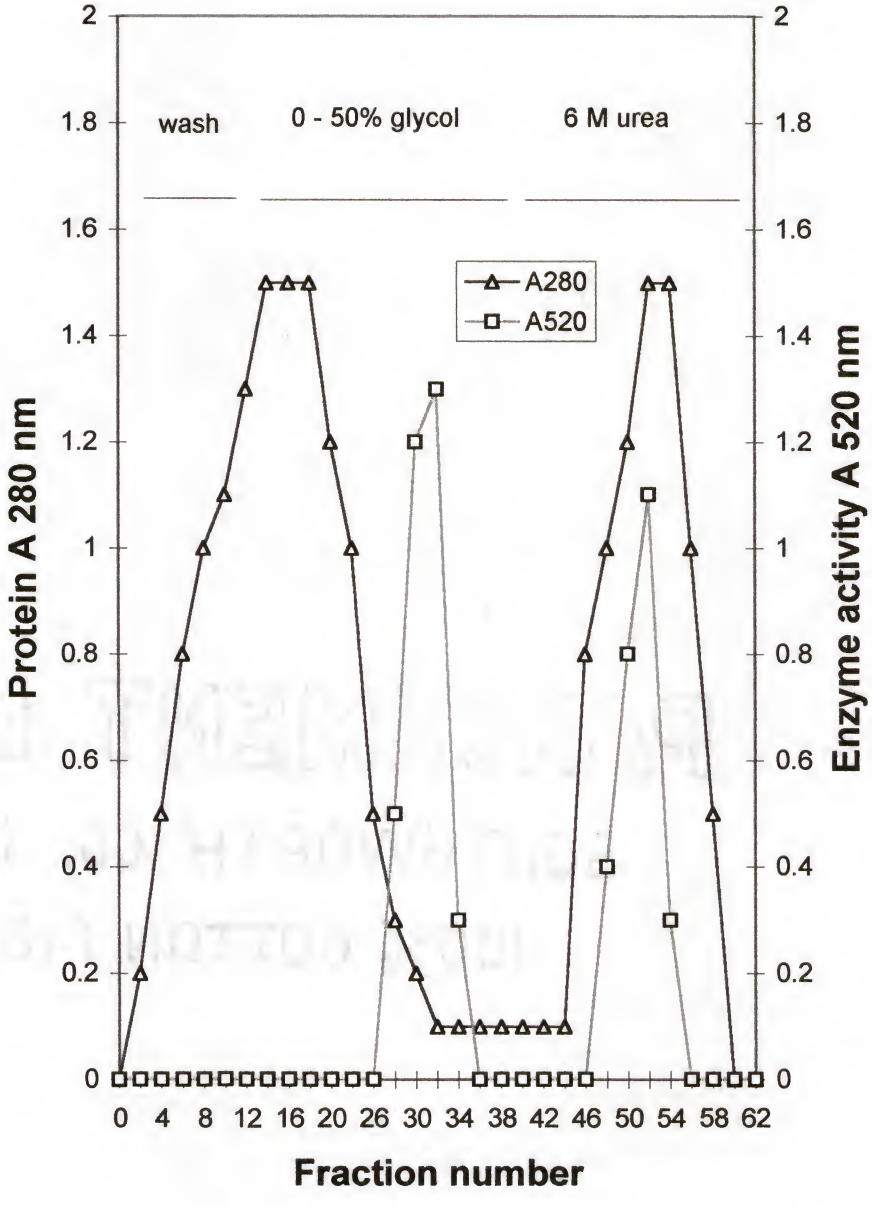

wash
0 - 50% glycol
6 M urea
A280
A520
Protein A 280 nm
Enzyme activity A 520 nm
Fraction number
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0 4 8 12 16 20 26 30 34 38 42 46 50 54 58 62

Fig. 23. Cation exchange chromatography of the β-(1->3)-glucanase. Activity from the glycol elution of Fig. 22 was applied to CM-Sepharose column, and eluted with a linear gradient of 0 - 0.5 M NaCl in buffer A. Absorbance at 280 nm is protein, and 520 nm is β-(1->3)-glucanase activity.

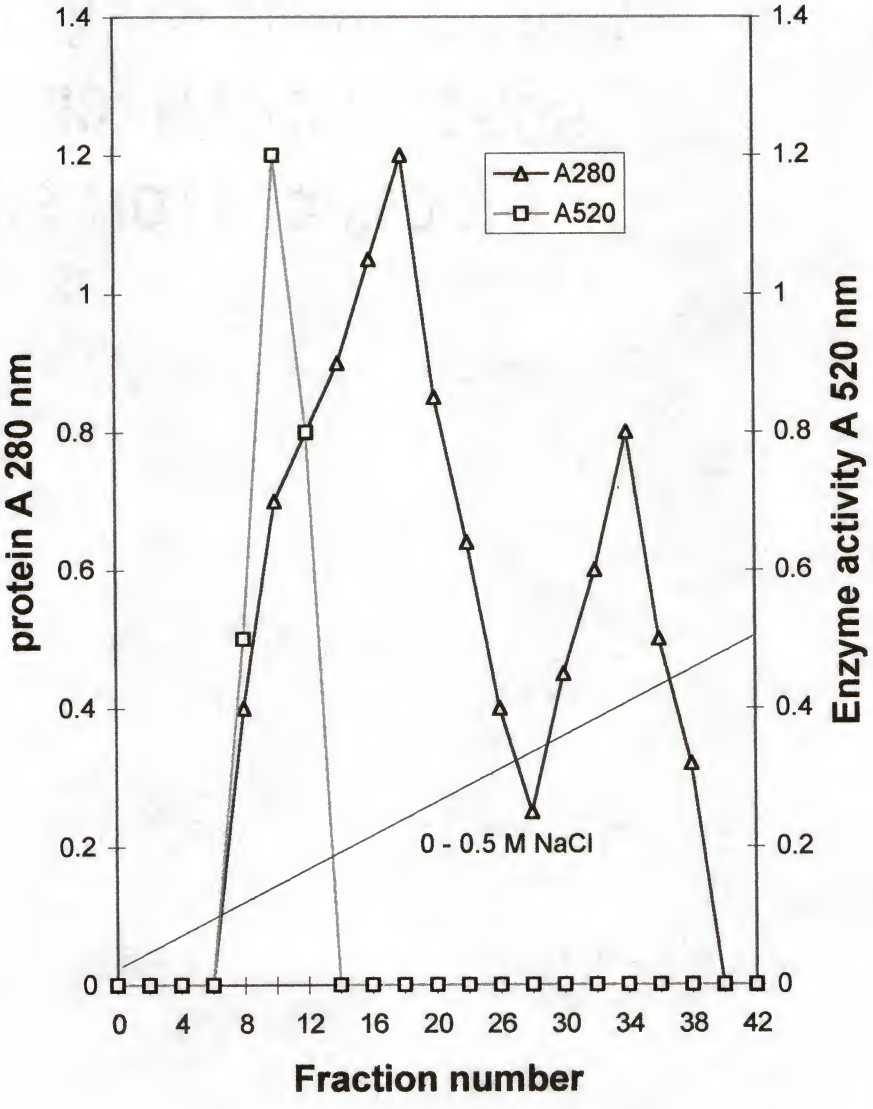

1.4
1.2
1
0.8
0.6
0.4
0.2
0
A280
A520
0 - 0.5 M NaCl
protein A 280 nm
Enzyme activity A 520 nm
0 4 8 12 16 20 26 30 34 38 42
Fraction number

Fig. 24. Anion exchange chromatography of the β-(1->3)-glucanase. Unbound activity from the Cibacron blue 3 GA column was applied to DEAE-cellulose column, and eluted with a linear gradient of 0 - 0.5 M NaCl in buffer B. Absorbance at 280 nm is protein, and 520 nm is β-(1->3)-glucanase activity.

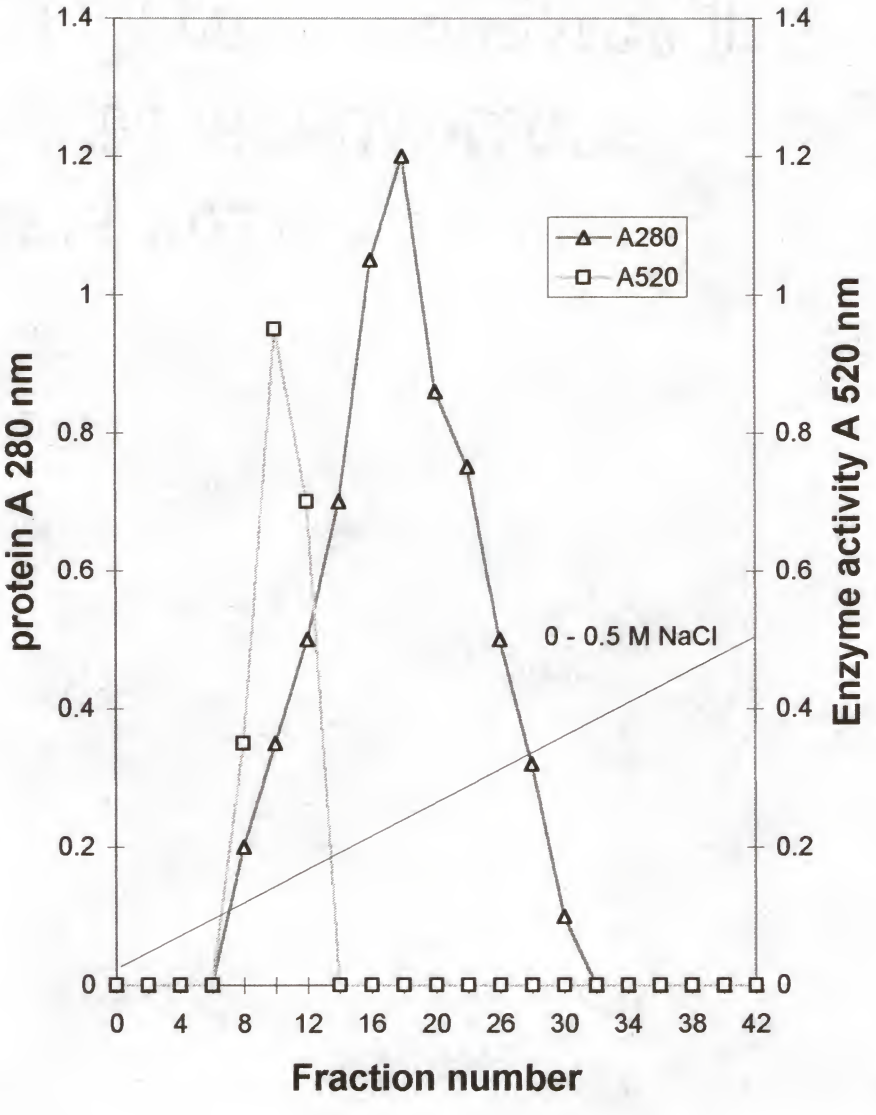

1.4
1.2
1
0.8
0.6
0.4
0.2
0
protein A 280 nm
Enzyme activity A 520 nm
A280
A520
0 - 0.5 M NaCl
0 4 8 12 16 20 26 30 34 38 42
Fraction number

Fig. 25. Gel filtration chromatography of the β-(1->3)-glucanase. Activity from the DEAE-cellulose column was applied to Sephadex G-200 column, and eluted with buffer B. Absorbance at 280 nm is protein, and 520 nm is β-(1->3)-glucanase activity.

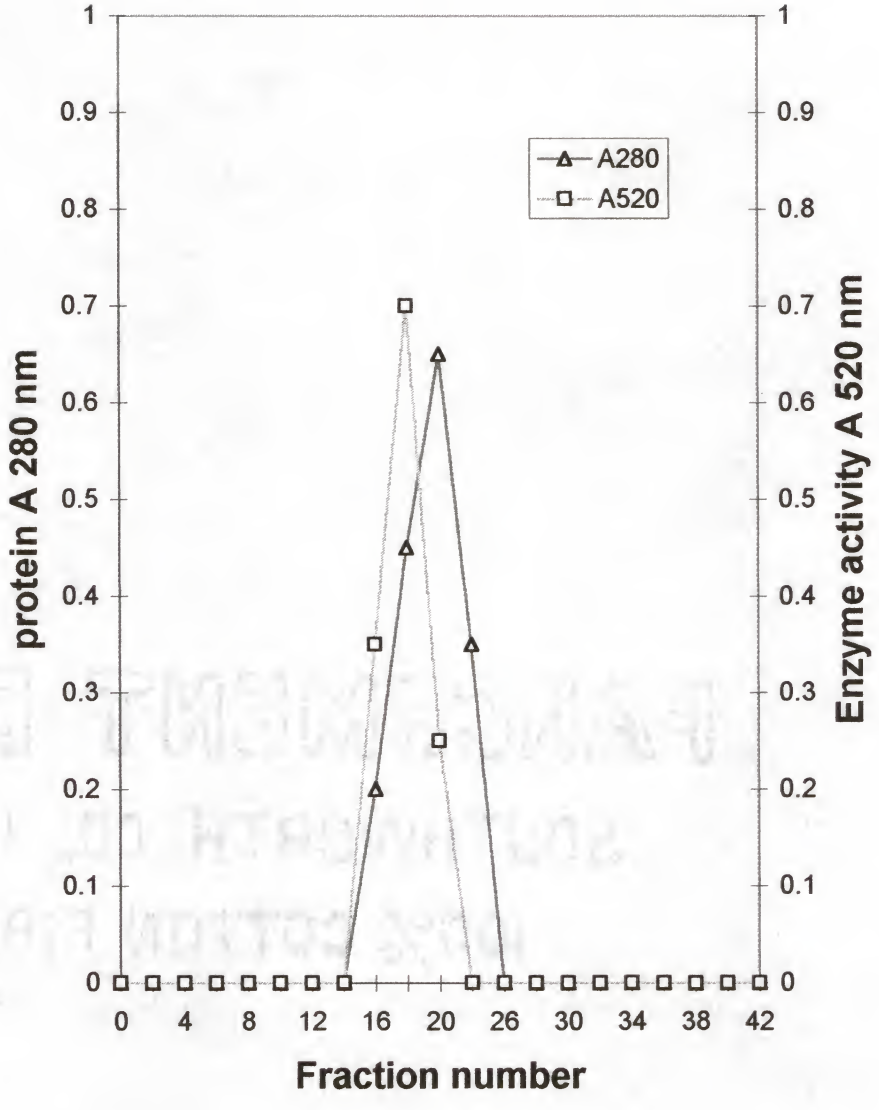

A280
A520
protein A 280 nm
Enzyme activity A 520 nm
Fraction number
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
4
8
12
16
20
26
30
34
38
42

Fig. 26. SDS-PAGE of proteins during purification of the β-(1->3)-glucanase. proteins on the gel were stained with Zinc Stain (Bio-RAD). Lane 1, glycol fractions from the phenyl-Sepharose column; lane 2, fractions eluted from CM-Sepharose column with NaCl; lane 3, fractions eluted from DEAE-cellulose column with NaCl; lane 4, fraction from Sephadex G-200 column; lane 5, standard protein markers from Sigma.

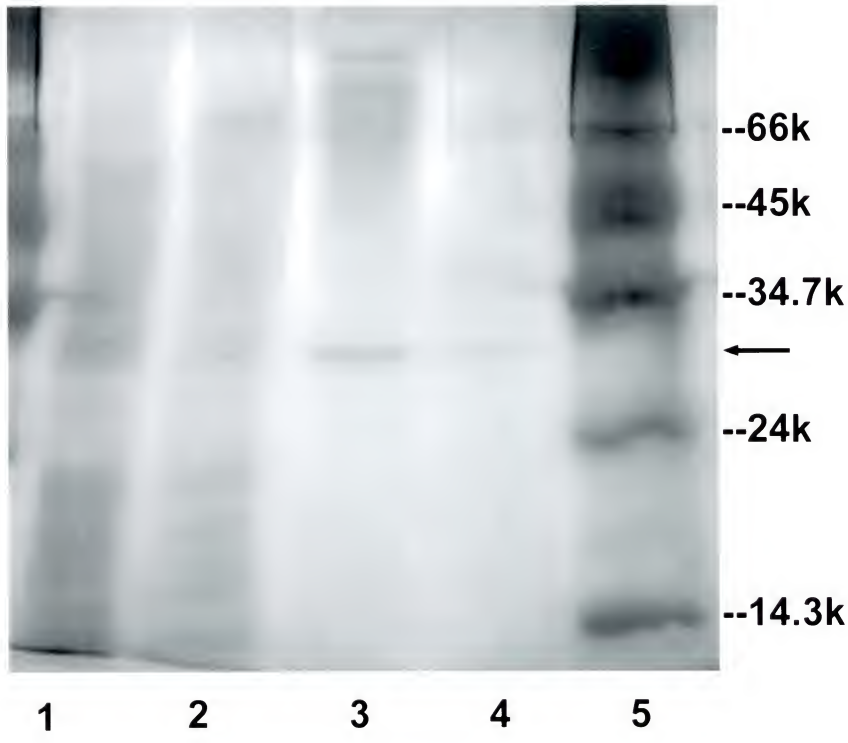

--66k
--45k
--34.7k
--24k
--14.3k
1
2
3
4
5

Fig. 27. The effect of pH on the activity and stability of the β-(1->3)-glucanase. The activity was assayed under standard condition at various pHs for 1 h. For pH stability, the enzyme was incubated in various pH levels at 4 C or 25 C for 24 h, the activity was then assayed.

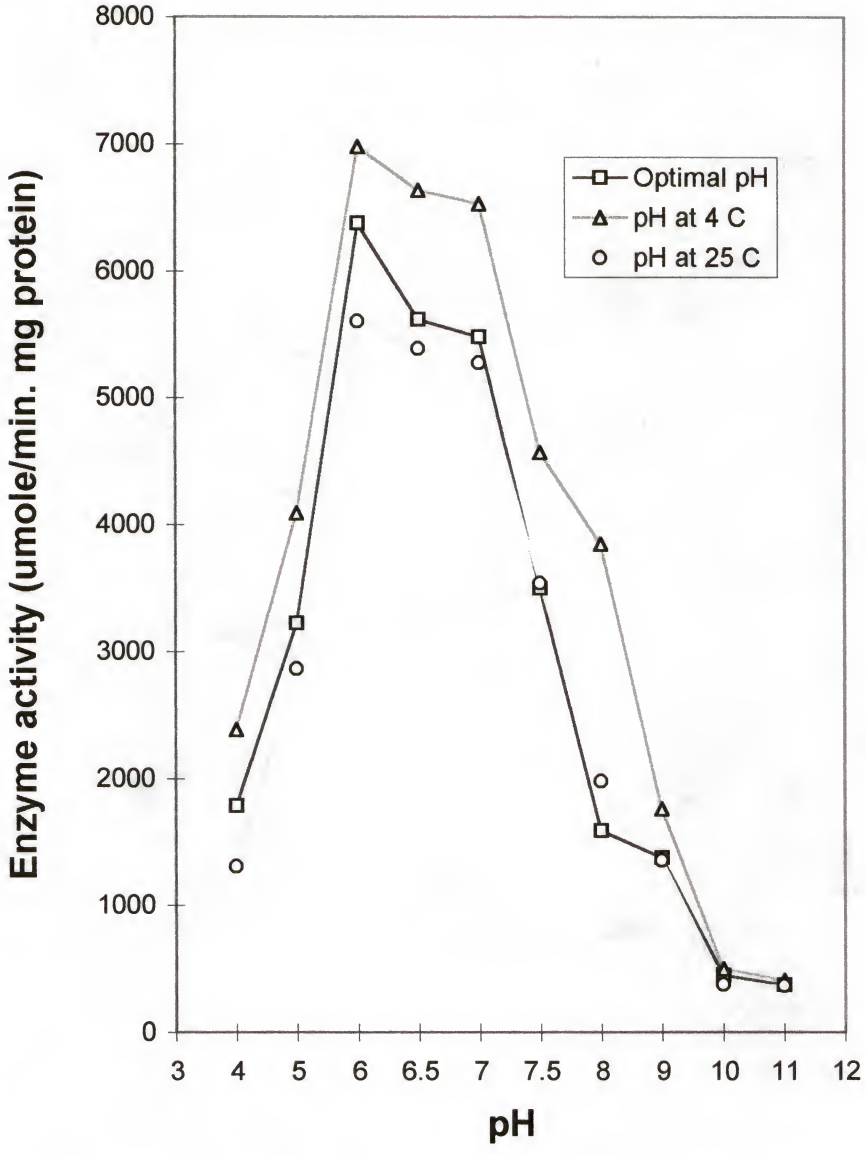

8000
7000
6000
5000
4000
3000
2000
1000
0
Enzyme activity (umole/min. mg protein)
Optimal pH
pH at 4 C
pH at 25 C
3
4
5
6
6.5
7
7.5
8
9
10
11
12
pH

Fig. 28. The effect of temperature on the activity and stability of the β-(1->3)-glucanase. The enzyme was assayed under standard condition in the range of 20 70 C for 1 h. For temperature stability, the enzyme was incubated in various temperature levels for 1 h, then assayed as given.

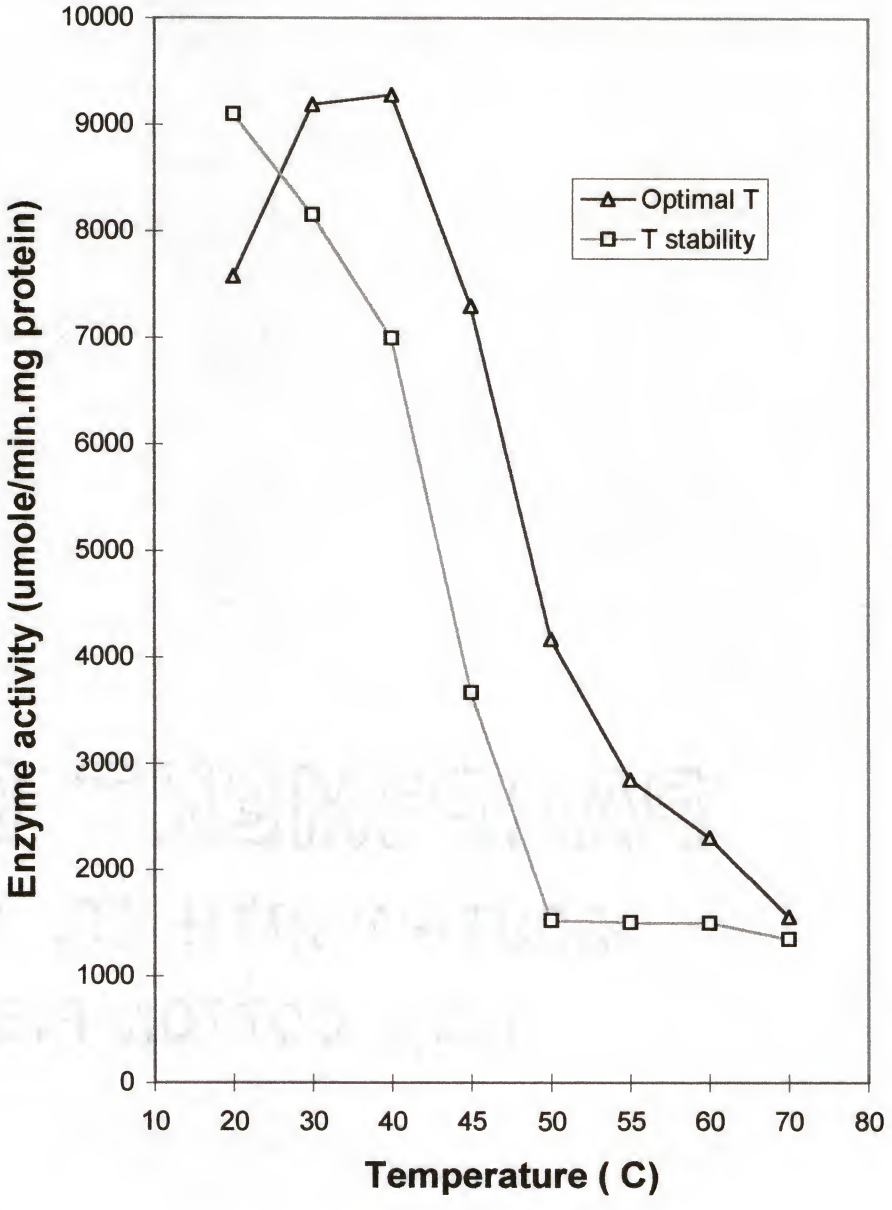

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Enzyme activity (umole/min.mg protein)
Optimal T
T stability
10
20
30
40
45
50
55
60
70
80
Temperature (C)

Table 5. The effect of metal ions and protease inhibitors on the purified β-(1->3)-glucanase

	Relative rate (%) [a]		
	0.1 mM	1 mM	5 mM
None	100	100	100
$CaCl_2$	102.1	111.5	101.2
$MgCl_2.6H_2O$	101.5	112.6	89.4
$MnCl_2.4H_2O$	92.3	96.4	57.7
$CoCl_2$	82.4	93.3	48.5
$LaCl_3.7H_2O$	72.8	75.0	30.7
$FeSO_4.7H_2O$	44.5	33.0	0
$ZnCl_2$	37.6	34.4	0
$FeCl_3$	14.6	13.5	0
$HgCl_2$,	14.3	12.4	0
$CuSO_4.5H_2O$	6.8	6.7	0
Protease inhibitors	100	92.0	64.0

[a] The rate without ions (8702 μmol min^{-1} mg^{-1} protein) was set at 100%, and all other rates were compared to this value.

Table 6. Relative rates of hydrolysis of β-glucans by the purified β-(1->3)-glucanase

Substrates	Main linkage type	Relative rate (%) [a] Reducing sugar [b]	Glucose [c]
Laminarin	β-(1->3)	100	100
Neutral glucan	β-(1->3)	66.1	58.5
Phosphoglucan	β-(1->3)	15.5	12.5
Crude glucan	β-(1->3)	23.6	18.1
Laminaribiose	β-(1->3)	/[d]	9.1
Gentiobiose	β-(1->6)	/	0
Cellulobiose	α-(1->4)	/	0
Cellulose	α-(1->4)	0	/
Lichenan	β-(1->4) / (1->3)	0	/
Schizophyllan	β-(1->3)	0	/
Pachyman	β-(1->3)	0	/
Pustulan	β-(1->6)	0	/
Pullulan	α-(1->6)	0	/

[a] The rate (8649 μmol min^{-1} mg^{-1} protein) for reducing sugar on laminarin was set at 100%, all other rates were compared to this value.

[b] Measured by Somogyi's method with glucose as the standard.

[c] Measured by the glucose oxidase-peroxidase method with glucose as the standard. 100 % correspond to 6496 μmol min^{-1} mg^{-1} protein for glucose.

[d] not measured.

Fig. 29. Chromatogram of the β-(1->3)-glucanase hydrolysate following various reaction times of laminarin on 5 X 20 cm pre-coated silica gel plate (Fisher). A sample of 10 μL was applied to the plate. The solvent system was n-butanol-acetic acid-ethyl ether-water (9:6:3:1, v/v). Phenol-sulfuric acid reagent was used to detect sugars. Lane 1, hydrolysate after 20 min.; lane 2, after 60 min.; lane 3, mixture ofglucose (GLC), laminaribiose (L2) and gentiobiose (G2); lane 4, after 12 h and lane 5, after 24 h.

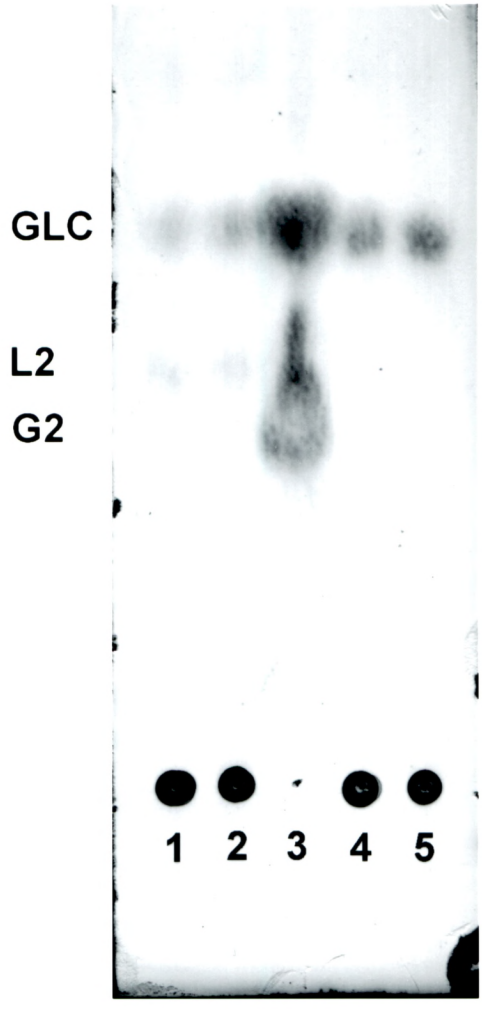

GLC
L2
G2
1
2
3
4
5

Fig. 30. Chromatogram of the β-(1->3)-glucanase hydrolysate following various reaction times of neutral glucan on 5 X 20 cm pre-coated silica gel plate (Fisher). Experimental method the same as Fig. 29. Lane 1, hydrolysate after 20 min.; lane 2, after 60 min.; lane 3, mixture of glucose (GLC), laminaribiose (L2) and gentiobiose (G2); lane 4, after 12 h and lane 5, after 24 h.

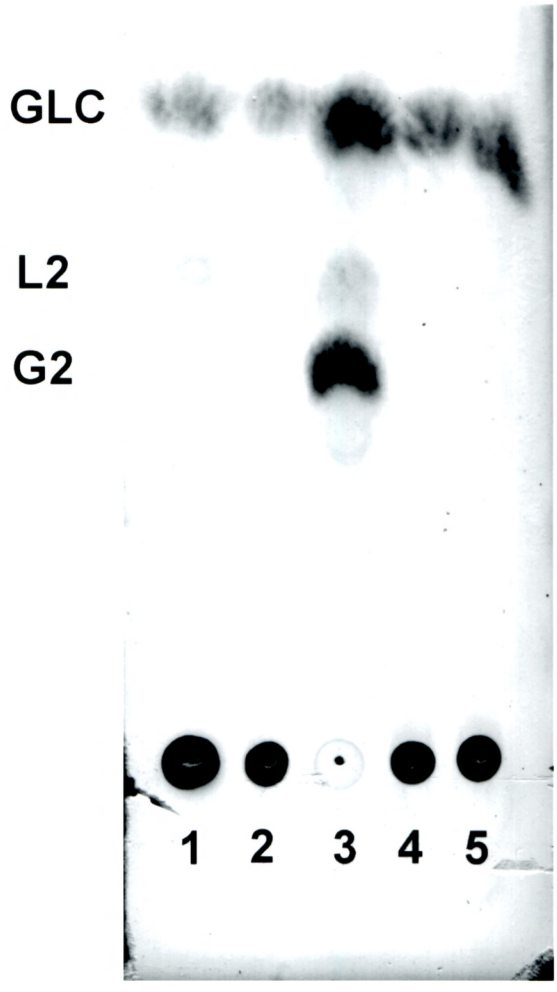

GLC
L2
G2
1
2
3
4
5

Fig. 31. Chromatogram of the β-(1->3)-glucanase hydrolysate following various reaction times of phosphoglucan on 5 X 20 cm pre-coated silica gel plate (Fisher). Experimental method the same as Fig. 29. Lane 1, hydrolysate after 20 min.; lane 2, after 60 min.; lane 3, mixture of glucose (GLC), laminaribiose (L2) and gentiobiose (G2); lane 4, after 12 h and lane 5, after 24 h.

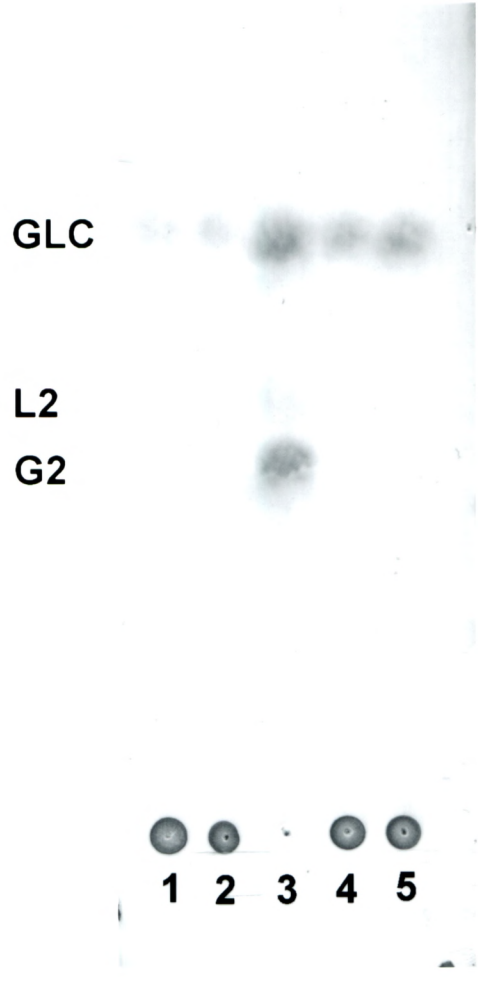
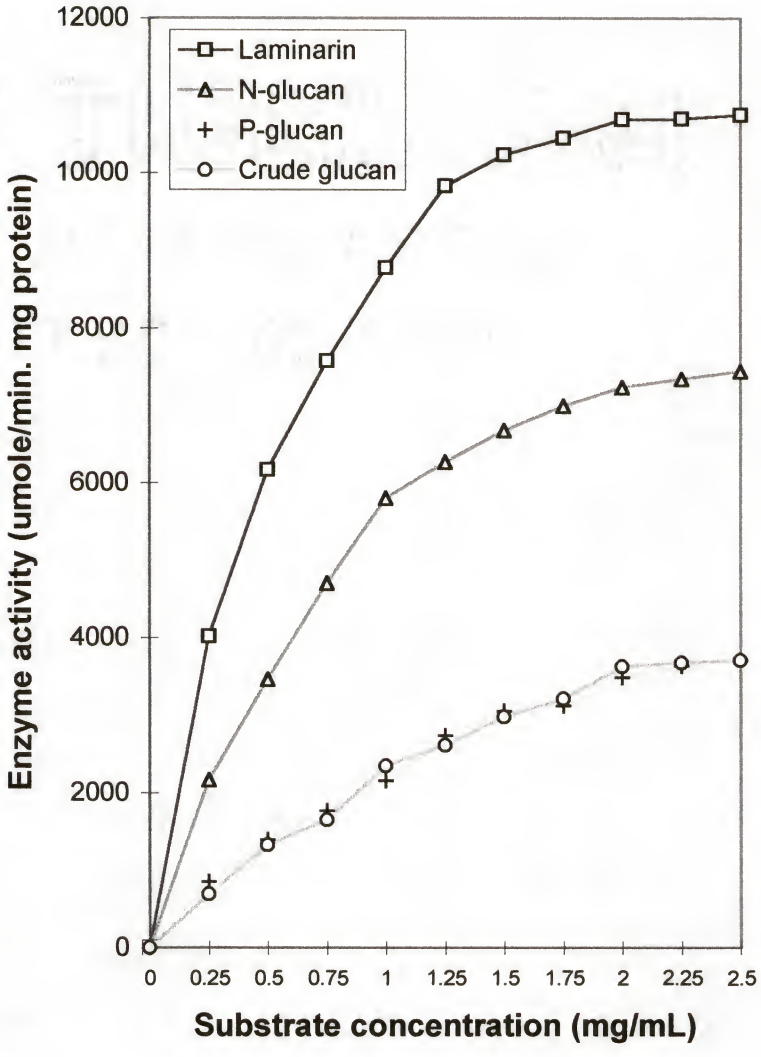

GLC
L2
G2
1 2 3 4 5

Fig. 32. The effect of concentration using several different substrates on the activity of the β-(1->3)-glucanase. Laminarin, neutral glucan, phosphoglucan and crude glucan were prepared in 50 mM sodium acetate buffer, pH 6 at the range of 0.25 - 2.5 mg mL^{-1}. Activity was assayed under standard assay condition for 1 h.


Laminarin
N-glucan
P-glucan
Crude glucan
Enzyme activity (umole/min. mg protein)
12000
10000
8000
6000
4000
2000
0
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
2.25
2.5
Substrate concentration (mg/mL)

Discussion

Purification of the β-(1->3)-glucanases activities from mycelial homogenates of Achlya during Ca^{2+}-induced sporulation was attempted using a series of chromatography procedures. Two major activities were found and one of them was apparently purified to homogeneity as judged by SDS-PAGE electrophoresis.

The purified β-(1->3)-glucanase, which constitutes about 60 % of the total crude mycelial β-(1->3)-glucanase activity, was studied in some detail. It has a molecular weight of about 30 kDa, which is similar to other exo-β-(1->3)-glucanases reported from fungi (Abd-El-Al and Phaff, 1968; Kitamoto et al., 1987), and at the low end of the range of 20 - 80 kDa reported for filamentous fungi (Piston et al., 1993).

The pH optimum of this enzyme is 6 (Fig. 27), which is similar to that of other fungi (Bielecki and Galas, 1991; Stone and Clarke, 1992; Piston et al., 1993; 1995; Mishra and Robbins, 1995). The bell-shaped activity curve may suggest that catalysis is mediated by two amino acid residues as shown in similar enzymes (Akiyama et al., 1997). In contrast to other β-(1->3)-glucanases, the activity has a narrow pH profile, and decreases rapidly in the basic range. The fact that activity increases about 10 % after storing at 4 C for 24 h may suggest that after purification and concentration procedures, this enzyme needs to return to its best conformation for catalysis.

The optimal temperature for activity is 40 C (Fig. 28), and this value is similar to that of the majority of fungal β-(1->3)-glucanases (Bielecki and Galas, 1991; Stone and Clarke,

1992; Piston et al., 1993; 1995; Mishra and Robbins, 1995). Although the activity is sensitive to temperatures over 55 C, it is stable at 4 C, losing only 10.5 % of its activity after 7 days.

This enzyme is strongly inhibited by Cu^{2+}, Hg^{2+} and Fe^{3+} at 0.1 mM (Table 5). Similar results are reported for the enzymes from Rhizoctonia (Totsuka and Usui, 1986), Streptomyces (Kusama et al., 1986), Neurospora (Hiura et al., 1986) and Acremonium (Piston et al., 1995). Strong inhibition of the enzyme by Hg^{2+} suggests that thiol groups may be important for the enzyme structure (Bielecki and Galas, 1991). Low concentrations of Mg^{2+} and Ca^{2+} slightly enhance the activity, and the enzyme can tolerate Ca^{2+} up to 5 mM. The protease inhibitor cocktail which contains EDTA, DTT and PMSF did not reduce the enzyme activity at the concentration used.

The substrate specificity of the β-(1->3)-glucanase has been investigated using a range of linear, substituted and branched β-glucans (Table 6). This enzyme has a relatively narrow range of substrates, which includes the glucans of Achlya, algal laminarin and the dimer laminaribiose. The highest activity was against laminarin and the neutral glucan of Achlya. Low activity was found with the phosphoglucan of Achlya, and laminaribiose. It appears that the single chained laminarin or neutral glucan is more susceptible to the β-(1->3)-glucanase attack than the large, aggregated phosphoglucan.

The enzyme did not hydrolyze pachyman or schizophyllan, the latter β-glucan is highly branched with single β-(1->6)-glucosyl unit every third glucose residue, suggesting that the trimer with a branch is not recognized by the enzyme, as indicated for similar enzymes (Piston et al., 1995; Fontaine et al., 1997). Like most fungal exo-β-(1->3)-glucanase, this enzyme shows no activity against the mixed-linked β-(1->4/1->3)-glucans such as lichenan. No activity was detected against the β-(1->6)-linkage or any type of α-linkage (Table 6).

The lack of significant activity against laminaribiose suggests that the enzyme requires a certain glucosyl residue length for enzyme-substrate binding. The suggested oligomer range for an exo- β-(1->3)-glucanase is 5 -8 glucosyl residues for significant activity (Akiyama et al., 1997). The activity from Trichoderma exhibits a linear increase in activity with an increase in oligomer length from laminaritetraose to laminarihexaose (Kitamoto et al., 1987). Similar results are reported from other organisms (Bielecki and Galas, 1991; Fontaine et al., 1997).

The pattern of hydrolysis for the β-(1->3)-glucanase was determined by examination of the products released from the hydrolysis of laminarin, neutral glucan and phosphoglucan on TLC. The pattern is that of an exo cleavage, since the initial products were glucose and a trace amount of laminaribiose. Additional incubation time gave glucose as the only product (Figs. 29 - 31), which is typical for most exo β-(1->3)-glucanases (Bielecki and Galas, 1991; Piston et al., 1995; Mishra and Robbins, 1995).

The kinetic analysis of the β-(1->3)-glucanase using laminarin, crude glucan, neutral glucan and phosphoglucan of Achlya allowed a calculation of K_m and V_{max} values. The K_m value of 0.67 mg mL^{-1} for laminarin is similar to values reported from other β-(1->3)-glucanases (Bielecki and Galas, 1991; Piston et al., 1995; Mishra and Robbins, 1995). The K_m value for neutral glucan 1.11 mg mL^{-1}, while that of phosphoglucan and crude glucan are much higher. K_m value reflects the binding affinity of substrate to enzyme, which is affected by the size, the degree of branching, substitution, and solubility of the substrates. The lower affinity of insoluble substrates seems a common enzymetic property for β-(1->3)-glucanase activities (Wong and Maclachlan, 1979; Notario, 1982). The four substrates used here all have high solubility in water, but phosphoglucan is very large, and cross-linked with

phosphodiesters, whereas neutral glucan is similar in both size and branching to laminarin (Lee et al., 1996). Thus, the molecular size and the presence of phosphate may contribute to the different K_m values found.

In this study, I have attempted to demonstrate β-(1->3)-glucanases capable of hydrolyzing the cytoplasmic β-(1->3)-glucans in order to support Ca^{2+}-induced sporulation. It was previously shown that during Ca^{2+}-induced sporulation, these β-(1->3)-glucans declined significantly (Cottingham and Mullins, 1985; Lee and Mullins, 1994), and it is now clear that the β-(1->3)-glucanases are responsible for this decline.

Chapter 6
UTILIZATION OF THE CYTOPLASMIC β-(1->3)-GLUCANS OF ACHLYA DURING CA^{2+}-INDUCED SPORULATION

Introduction

Sporulation provides a convenient system for the investigation of carbohydrate metabolism and the utilization of reserve carbohydrates. In yeast reserve carbohydrates synthesized early in development were completely degraded prior to spore formation, suggesting them as a source of energy and carbon to support sporulation (Rothman and Cabib, 1969; Becker et al., 1988; Katohda et al., 1988; Rua et al., 1993). In Achlya, β-(1->3)-glucans accumulated in the cytoplasm during growth then decreased by 1/3 during asexual sporulation, indicating a similar role in spore formation (Cottingham and Mullins, 1985; Lee and Mullins, 1994).

Thus the biological role of reserve carbohydrates seems clear except for the phosphorylated ones and the role of phosphate. In this chapter, the roles of both carbon and phosphate in cytoplasmic reserves will be investigated.

Materials and Methods

Culture of Organism

See Chapter 3 for basic cultural techniques.

Extraction, Fractionation and Gel Filtration of Glucans

Mycelium was harvested at 0, 4, 8 and 12 h after sporulation, washed and frozen. The frozen mycelium was then lyophilized for 48 h and weighed. The extraction, fractionation and gel filtration of glucans followed the method used by Lee and Mullins (1994).

NMR Study of Phosphoglucan

^{32}P NMR spectra were recorded using a Nicolet 70.5 -kG multinuclear tunable Fourier-transform spectrometer operating at 121.5 MHz in the pulsed Fourier transformed mode, with a spectral window of 5000 Hz, pulse width, 20 μs and an acquisition time of 500 ms. The pH of the sample was adjusted to 7.5. Phosphorus-31 chemical shifts are referenced to an internal p-nitrophenylphosphorylcholine at -5.55 ppm. This instrument is located in Department of Microbiology and Cell Science, under the supervision of Dr. John Gander.

Analytic Methods

Total Carbohydrate

Total carbohydrate was determined by the phenol-sulfuric acid method (Dubois et al., 1956). A sample of 0.2 mL and 0.2 ml of 5 % (v/v) phenol were mixed before 1 mL concentrated sulfuric acid was added. After cooling to room temperature, absorbance was read at 488 nm. Glucose was used as the standard.

Total and Inorganic Phosphate

Total phosphate and inorganic phosphate were measured by the Ames method (1966). For total phosphate, a sample of 0.01 - 0.1 mL was mixed with 0.3 ml of 10 % $Mg(NO_3)_2 . 6H_2O$. The solution was ashed by shaking the tube over a strong flame until the brown color disappeared. After cooling, 0.3 ml of 0.5 N HCl was added to the tube, followed by heating in boiling water bath for 15 min to hydrolyze any pyrophosphate to phosphate. After cooling, 0.7 ml of a fresh mixture containing 10 % ascorbic acid and 0.42 % ammonium molybdate in a ratio of 1:6 (v/v) was added, then incubated at 45 C for 20 min, the resulting absorbance was measured at 820 nm with a red filter. When measuring inorganic phosphate, 0.7 mL of the 10 % ascorbic acid and 0.42 % ammonium molybdate mixture is initially added to the sample, followed by incubation and measurement.

Results

Fractionation of the Cytoplasmic Glucans

The fractionation of crude glucans was performed by the batch method with Macro-Prep 50 Q anion exchange gel. This method was rapid, gave good recovery, and Fig. 33 shows a typical elution pattern of crude glucan. Neutral glucan was not bound and was eluted with the buffer. Bound phosphoglucan was then eluted with salt.

Utilization of Glucans During Sporulation

The effect of sporulation on mycelial weight and reserve cytoplasmic glucan of Achlya were summarized on Table 7. The mycelial glucan decreased dramatically 4 h into sporulation, and mycelial weight showed a small decrease. After 12 h, glucan continued to decrease and mycelial weight declined significantly.

Both neutral glucan and phosphoglucan declined during sporulation. Their ratio changed, with the percentage of neutral glucan increasing with increasing time of sporulation. The phosphate content of phosphoglucan declined throughout sporulation (Table 8).

Gel Filtration Chromatography of Phosphoglucan

Gel filtration chromatography profiles of the phosphoglucan during the time course of sporulation were similar (Fig. 34). It eluted just after the void volume and showed a broad distribution. With increasing time of sporulation, the peak fraction gradually decreased and shifted down the gradient.

NMR Study of the Phosphoglucan

Phosphorus-31 NMR spectra of the phosphoglucan are presented on Figs. 35-38. All spectra were similar with the phosphodiester peak at ~ 1 ppm and the phosphomonoester peak at ~ 4.5 ppm. At 0 h of Ca^{2+}-induced sporulation, the monoester peak was smooth, but at 4, 8 and 12 h into sporulation, small shoulder peaks appeared.

The ratio of monoester to diester linkages are summarized in Table 9. The ratio at 0 h was 80 : 20, while the ratios at 4, 8 and 12 h were similar with a slight increase in the diester linkage.

Fig. 33. Batch fractionation of mycelial glucan from Achlya on Macro-Prep 50 Q. Neutral glucan was eluted with 5 mM Tris-HCl buffer, pH 7.6. The retained component, phosphoglucan, was eluted with 0.6 M NaCl in the same buffer. carbohydrate content was measured by the phenol-sulfuric acid method.

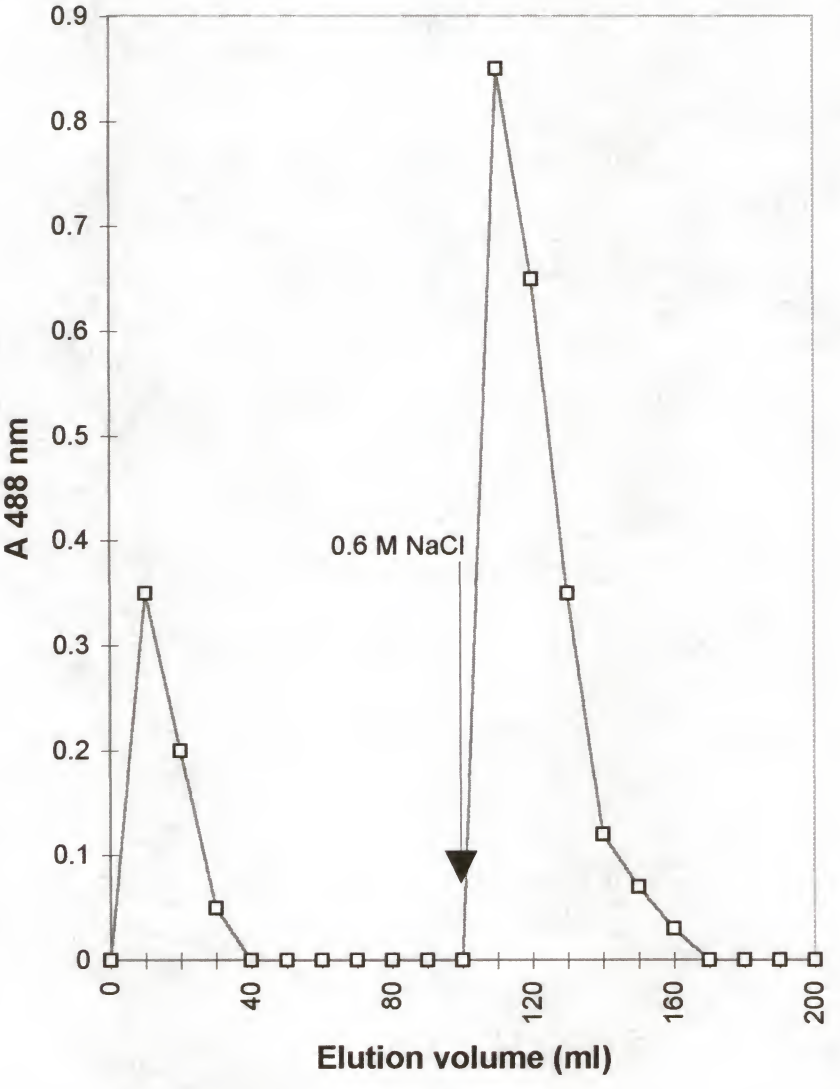

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A 488 nm
0.6 M NaCl
0
40
80
120
160
200
Elution volume (ml)

Table 7. Effect of sporulation on mycelial weight and glucan of Achlya

Sporulation time (h)	Mycelial dry weight (mg flask^{-1})[a]	change(%)[b]	Mycelial glucan (mg flask^{-1})[a]	change(%)[b]
0	240.67 ± 19.0	0	33.4 ± 2.8	0
4	224.7 ± 5.2	-6.63	19.13 ± 0.7	-42.6
8	212.8 ± 9.4	-11.7	16.88 ± 1.33	-49.4
12	188.5 ± 4.2	-21.7	12.51 ± 0.5	-62.6

[a] Numbers are the average ± SD of three individual flasks grown on MEM for 36 h, then subjected to Ca^{2+}-induced sporulation.
[b] As compared with 0 h.

Table 8. Glucan and phosphate utilization of Achlya during induced sporulation

Sporulation time (h)	Mycelial glucan Neutral glucan (mg flask^{-1})[a]	% [b]	Phosphoglucan (mg flask^{-1})[a]	% [c]	Total phosphate % (w/w)[d]
0	7.37 ± 0.5	21.8	26.5 ± 2.1	78.2	7.31 ± 0.23
4	4.33 ± 0.3	22.6	14.8 ± 2.2	77.4	5.27 ± 0.25
8	4.68 ± 0.2	28.7	11.6 ± 0.7	71.3	4.00 ± 0.21
12	4.20 ± 0.3	33.2	8.47 ± 0.4	66.8	3.67 ± 0.15

[a] Numbers are the average ± SD of three individual flasks grown on MEM for 36 h, then subjected to Ca^{2+}-induced sporulation. Glucan was separated into neutral and phosphoglucan with Micro-Prep 50 Q anion exchanger.
[b] Percentage of neutral glucan/total glucan.
[c] Percentage of phosphoglucan/total glucan.
[d] Phosphate content of the phosphoglucan.

Fig. 34. Gel filtration of the phosphoglucans of Achlya from 0, 4, 8 and 12 h sporulation on P-60. Sample (2 mg mL^{-1}) was dissolved in water and loaded on a column of 1 x 48 cm. Elution with water and 1 ml fractions were collected. Carbohydrate content was measured by the phenol-sulfuric acid method. Void volume (Vo) was determined by elution of blue dextran (molecular weight, 2,000,000).

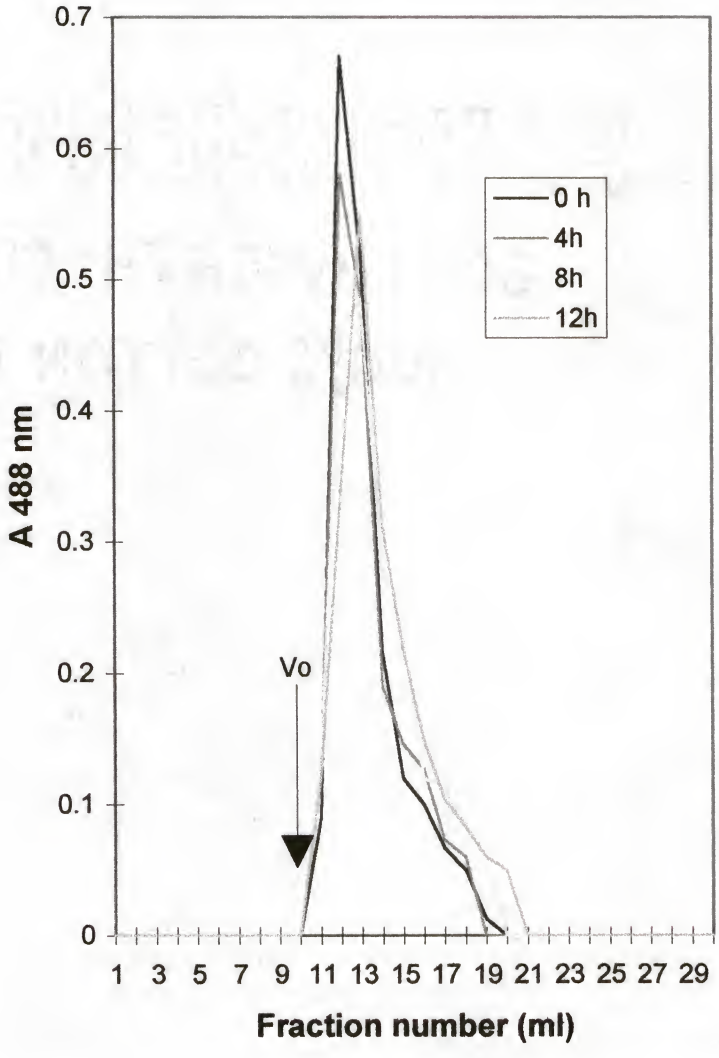

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A 488 nm
0 h
4h
8h
12h
Vo
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Fraction number (ml)

Fig. 35. Proton-decoupled phosphorus-31 NMR spectrum of phosphoglucan from 0 h sporulation. Sample (50 mg) was dissolved in deuterium oxide and adjusted to pH 7.5. Data acquisition parameters were sweep width, 5000 Hz; pulse width, 20 μs; acquisition time 500 ms; spectrometer frequency, 121.5 MHz; temperature, ambient (ca. 23 C). Spectrum represents 500 transients. Phosphorus-31 chemical shift are referenced to an internal p-nitrophenylphosphorylcholine at -5.55 ppm.

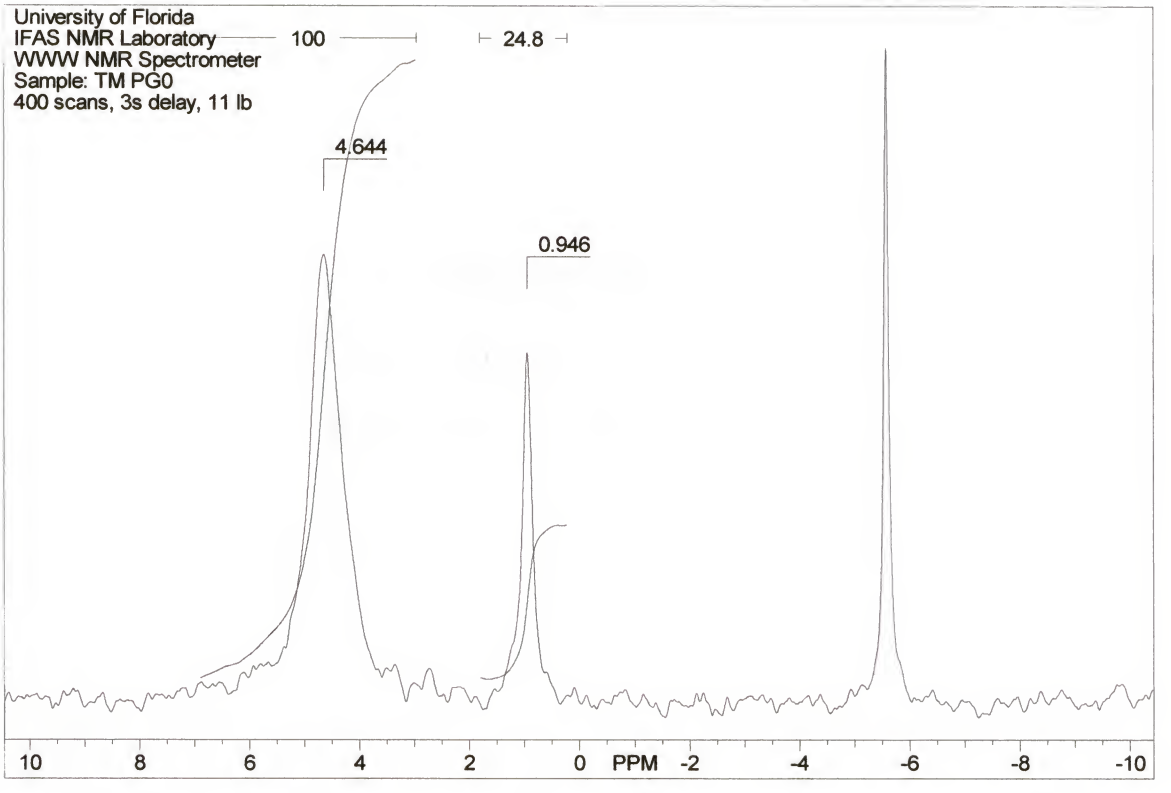

University of Florida
IFAS NMR Laboratory
WWW NMR Spectrometer
Sample: TM PG0
400 scans, 3s delay, 11 lb
100
24.8
4.644
0.946
10
8
6
4
2
0
PPM
-2
-4
-6
-8
-10

Fig. 36. Proton-decoupled phosphorus-31 NMR spectrum of phosphoglucan from 4 h sporulation. Experimental method was the same as Fig. 35.

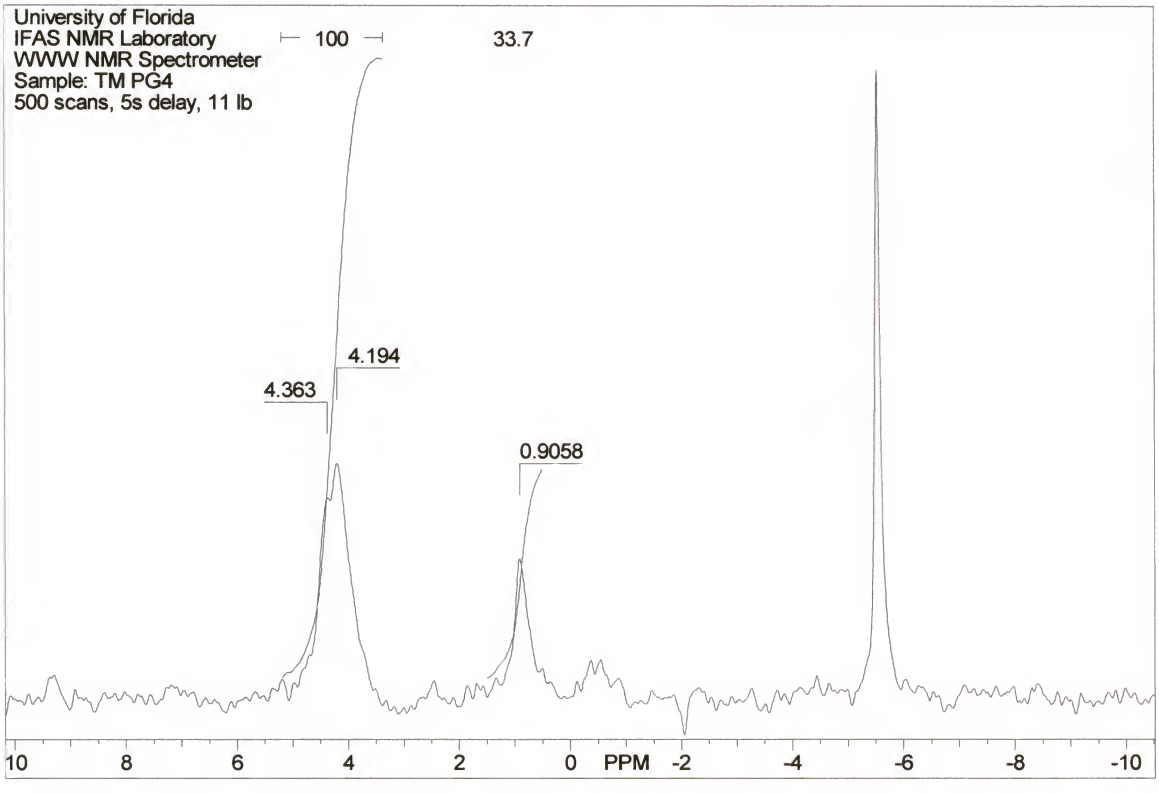

University of Florida
IFAS NMR Laboratory
WWW NMR Spectrometer
Sample: TM PG4
500 scans, 5s delay, 11 lb
100
33.7
4.194
4.363
0.9058
10
8
6
4
2
0
PPM
-2
-4
-6
-8
-10

Fig. 37. Proton-decoupled phosphorus-31 NMR spectrum of phosphoglucan from 8 h sporulation. Experimental method was the same as Fig. 35.

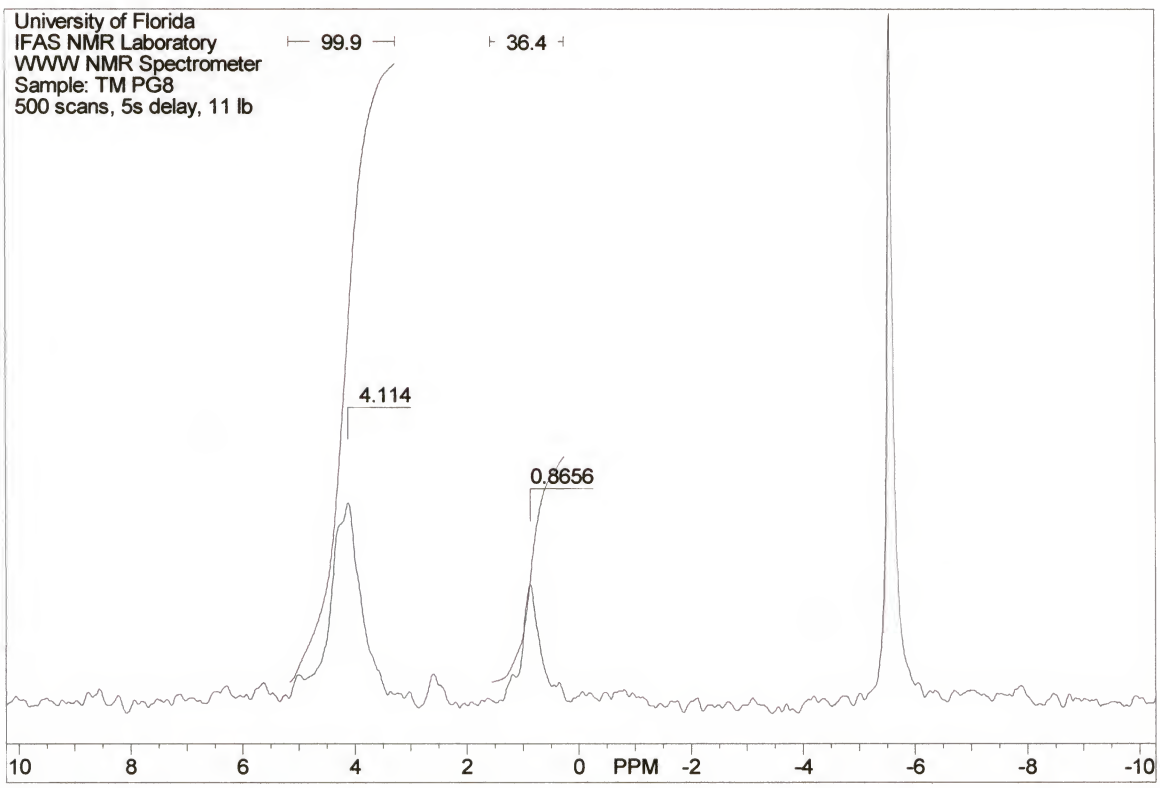

University of Florida
IFAS NMR Laboratory
WWW NMR Spectrometer
Sample: TM PG8
500 scans, 5s delay, 11 lb
99.9
36.4
4.114
0.8656
10
8
6
4
2
0
PPM
-2
-4
-6
-8
-10

Fig. 38. Proton-decoupled phosphorus-31 NMR spectrum of phosphoglucan from 12 h sporulation. Experimental method was the same as Fig. 35.

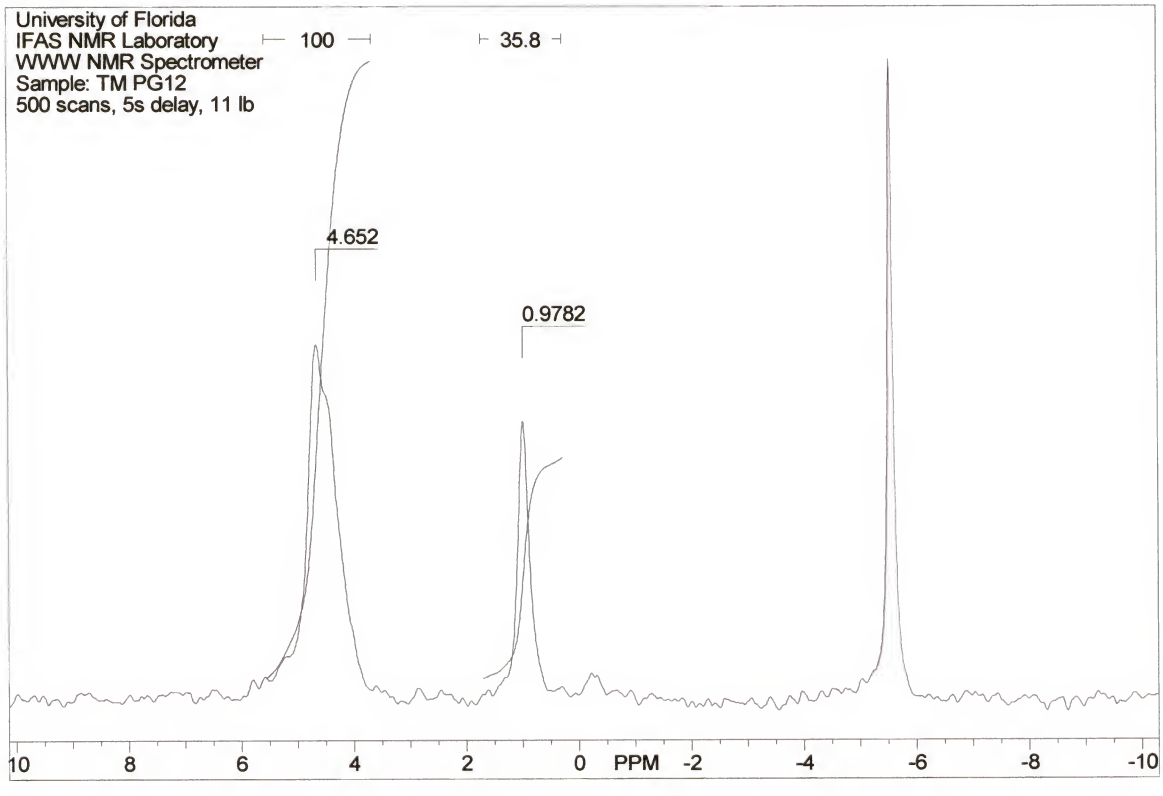

University of Florida
IFAS NMR Laboratory
WWW NMR Spectrometer
Sample: TM PG12
500 scans, 5s delay, 11 lb
100
35.8
4.652
0.9782
10
8
6
4
2
0
PPM
-2
-4
-6
-8
-10

Table 9. The ratios of mono- and di-ester linkages of phosphoglucan during sporulation of Achlya

Sporulation time (h)	Monoester (%)[a]	Diester (%)[a]
0	80.31	19.69
4	71.79	28.21
8	72.43	27.57
12	73.15	26.85

[a] The percentages were calculated according to the peak areas from the NMR spectra (Fig. 35-38).

Discussion

Many fungi accumulate reserve carbohydrates when the growth medium contains an excess of carbon. The most common form is polyol, either mannitol or arabitol, followed by the disaccharide trehalose and the polysaccharide glycogen (Blumental, 1976). In contrast, Oomycetes accumulate β-glucans in the cytoplasm (Faro, 1972; Cottingham and Mullins, 1985; Lee and Mullins, 1994). The traditional function of storage carbohydrates is to supply the organism with available carbon and energy under starvation conditions and in some fungi to support reproductive cycles (Blumental, 1976).

In Achlya, asexual sporulation can be induced by 0.5 mM Ca^{2+}, and requires about 6 h for completion, during that time the cytoplasmic β-glucans decreased by 33% (Cottingham and Mullins, 1985). A time course of a similar decrease in another species is showed in Table 7. After 4 h, mycelial glucan decreased by 42.6 %, while mycelial dry weight decreased by 6.63 %. Since the cytoplasmic β-glucans represent 13 % of mycelial dry weight (Lee and Mullins, 1994), this mycelial dry weight decrease is largely a decrease in the β-glucans. These results suggest that the cytoplasmic β-glucans were utilized to support sporulation, since the β-glucans decrease coincided with the appearance of sporangia and the formation of spores.

The cytoplasmic β-glucans can be separated into phosphoglucan and neutral glucan by anion exchange chromatography (Fig. 33). The molecular weight of the neutral glucan is estimated to 9000 with a dp of 50, when compared to algal laminarin (Lee et al., 1996). The molecular weight of phosphoglucan may be more than a million and is poorly resolved by gel filtration chromatography. During sporulation, both neutral glucan and phosphoglucan

decreased (Table 8), and the ratio of neutral glucan to phosphoglucan changed between 4 and 12 h. The percentage of phosphoglucan decreased with increasing time of sporulation, while that of neutral glucan increased (Table 8). Total phosphate content of the phosphoglucan decreased throughout sporulation, and more than half of the phosphate is removed (Table 8). A mechanism for this decrease is suggested by the increases of PDEase and PMEase activities (Figs. 9-10) as described in Chapter 3.

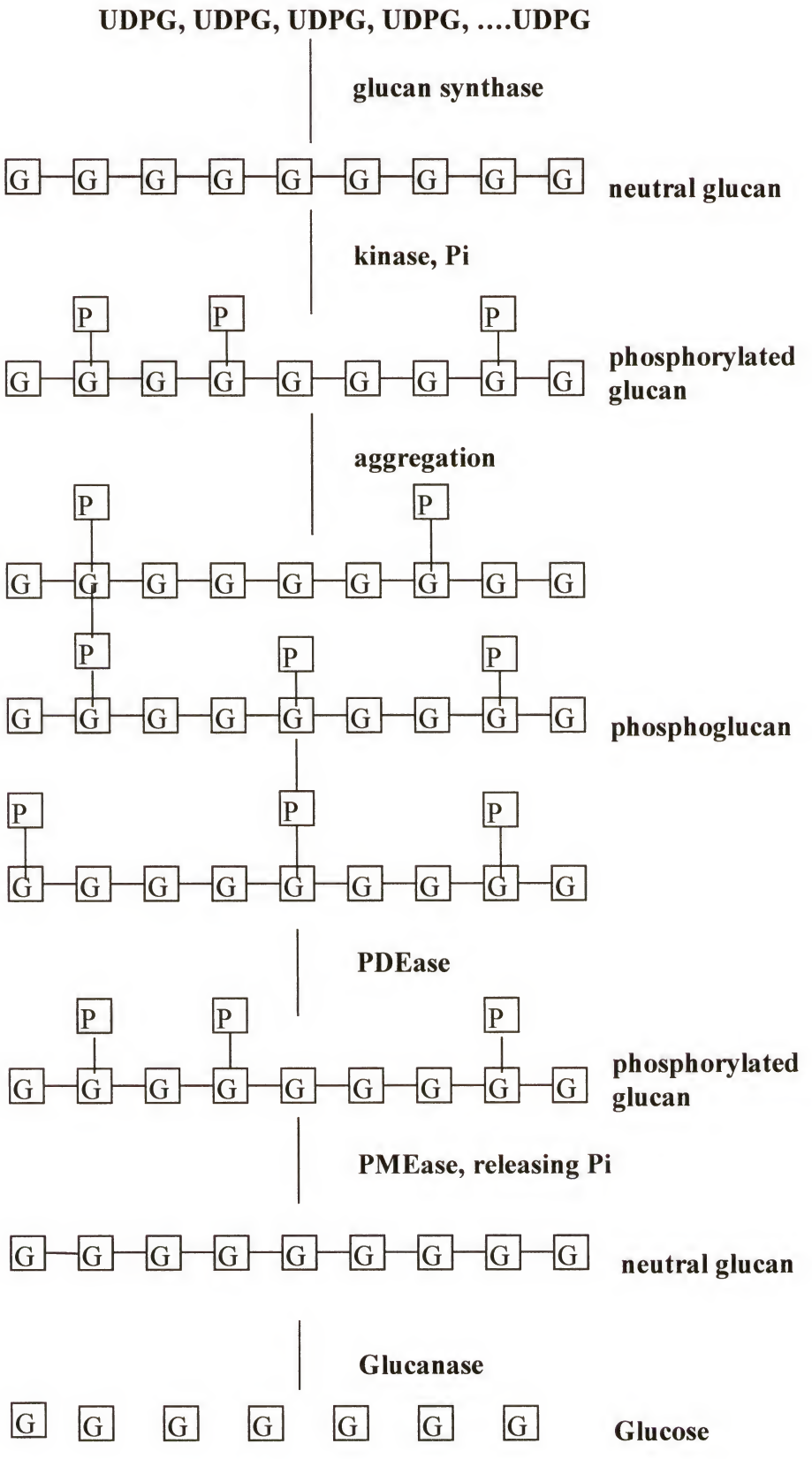
The results of the purification and characterization of the β-(1->3)-glucanase (Section III, Table 6) demonstrate that neutral glucan is the preferred substrate. Thus, a cooperative action between this glucanase and PDEase and PMEase is suggested, whereby PDEase action would result in a decrease in polymer size followed by PMEase activity to produce neutral glucan, the substrate for the glucanase. This is also supported by the dephosphorylation of phosphoglucan by HF, which converts the phosphoglucan into neutral glucan as shown by gel filtration data and ^{13}C-NMR spectra (Lee et al., 1996). Thus, they suggested that a cleavage of the diester linkage would result in a reduction in size of the phosphoglucan to that of the neutral glucan.

The gel filtration chromatography profiles of phosphoglucans during the time course of sporulation were similar (Fig. 34), suggesting again that the phosphatases and glucanases work concomitantly. Phosphorus-31 NMR spectra of the phosphoglucans were very similar (Figs. 35-38). Small shoulder peaks can be noticed for the monoester peaks at 4, 8 and 12 h, the cause of these peaks is not clear. The ratio of monoester : diester linkages at 0 h is 80 : 20 (Table 9), and during the course of sporulation, the ratios changed marginally. This supports the finding of gel filtration chromatography (Fig. 34) that no significant size change occurs in the phosphoglucan. We had originally thought that the phosphatases would convert

phosphoglucan into neutral glucan, and this would be shown by a reduction in size with gel filtration, and the loss of diester linkages and/or a change in the ratio of mono- to diester linkages with ^{31}P-NMR. This was not found, rather a simultaneous action of all three enzyme activities is shown.

These results clarify certain details of the utilization of cytoplasmic glucans in the support of spore formation. The process of sporulation only utilizes about 12 % of the mycelial dry weight during the first 8 h of Ca^{2+}-induction (Table 7). Total cytoplasmic β-glucans are included in this dry weight and they decline from 14 % of the initial value to 8 % over the same time period. The decline in total glucans for the 8 h period of sporulation is about 49 %. Phosphate is mobilized as the phosphodiester linkages are cleaved by phosphodiesterase and phosphomonoesterase action releases phosphate via dephosphorylation, thus producing neutral glucan which was shown to be the best substrate for the β-(1->3)-glucanase. The large reduction in phosphate content of the phosphoglucan also suggests that phosphate is made available for the biosynthesis of other molecules, such as nucleic acids, which are required for spore formation. The ratio of mono- and diester linkages remain similar to that of the vegetative mycelium, suggesting that there is no preferential cleavage of the diester bonds.

Fig. 39 Proposed diagram for the synthesis and degradation of the β-glucans



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BIOGRAPHICAL SKETCH

The author was born in Yibin county, Sichuan province, China, on May 10, 1962, where he grew up and received primary education. After graduating from high school in 1978, he entered Southwest Agricultural University, at Chongqing City, China. Seven years later, after receiving a BS degree in Agronomy and a master's degree in Plant Genetics, he became a faculty member at that university until 1989. He moved to Beijing, China working as a governmental official at the Agricultural Ministry of China from 1989 to 1990, then returned to Chongqing City to continue his research and teaching there. In 1991, he arrived at Gainesville, Florida to pursue his Ph. D degree in the Botany Department, University of Florida.

He is married to his wife, Lan Mu, and is anxiously waiting for their baby coming to the world.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John T. Mullins, Chairman
Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May, 1998

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